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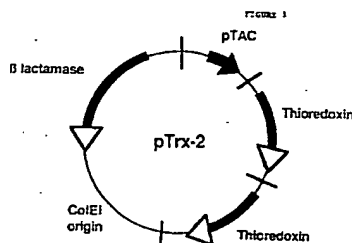
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(54) **DNA Sequencing.**

(57) The invention relates to methods of DNA sequencing including a method for determining the nucleotide base sequence of a DNA molecule, comprising providing the DNA molecule annealed with a primer molecule able to hybridize to the DNA molecule,

incubating the annealed mixture with a DNA polymerase, except reverse transcriptase, in the presence of four different deoxynucleoside triphosphates and a chain terminating agent which terminates DNA synthesis at a specific nucleotide base, the concentration of all four deoxynucleoside triphosphates at the start of said incubating being sufficient to allow DNA synthesis to continue until terminated by said agent. The DNA products of the incubating reaction are separated according to their size, so that at least a part of the nucleotide base sequence of the DNA molecule can be determined. In a further embodiment the primer may be extended and labelled prior to the incubating reaction.



This invention relates to DNA Sequencing

DNA sequencing involves the generation of four populations of single stranded DNA fragments having one defined terminus and one variable terminus. The variable terminus always terminates at a specific given nucleotide base (either guanine (G), adenine (A), thymine (T), or cytosine (C)). The four different sets of fragments are each separated on the basis of their length, on a high resolution polyacrylamide gel; each band on the gel corresponds colinearly to a specific nucleotide in the DNA sequence, thus identifying the positions in the sequence of the given nucleotide base.

Generally there are two methods of DNA sequencing. One method (Maxam and Gilbert sequencing) involves the chemical degradation of isolated DNA fragments, each labeled with a single radiolabel at its defined terminus, each reaction yielding a limited cleavage specifically at one or more of the four bases (G, A, T or C). The other method (dideoxy sequencing) involves the enzymatic synthesis of a DNA strand. Four separate syntheses are run, each reaction being caused to terminate at a specific base (G, A, T or C) via incorporation of the appropriate chain terminating dideoxynucleotide. The latter method is preferred since the DNA fragments are uniformly labelled (instead of end labelled) and thus the larger DNA fragments contain increasingly more radioactivity. Further, ³⁵S-labelled nucleotides can be used in place of ³²P-labelled nucleotides, resulting in sharper definition; and the reaction products are simple to interpret since each lane corresponds only to either G, A, T or C. The enzyme used for most dideoxy sequencing is the *Escherichia coli* DNA-polymerase I large fragment ("Klenow"). Another polymerase used is AMV reverse transcriptase.

Summary of the Invention

In one aspect the invention features a method for determining the nucleotide base sequence of a DNA molecule, comprising annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating separate portions of the annealed mixture in at least four vessels with four different deoxynucleoside triphosphates, a processive DNA polymerase wherein the polymerase remains bound to a DNA molecule for at least 500 bases before dissociating in an environmental condition normally used in the extension reaction of a DNA sequencing reaction, the polymerase having less than 500 units of exonuclease activity per mg of polymerase, and one of four DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base. The agent terminates at a different specific nucleotide base in each of the four vessels. The DNA products of the incubating reaction are separated according to their size so that at least a part of the nucleotide base sequence of the DNA molecule can be determined.

In preferred embodiments the polymerase remains bound to the DNA molecule for at least 1000 bases before dissociating; the polymerase is substantially the same as one in cells infected with a T7-type phage (i.e., phage in which the DNA polymerase requires host thioredoxin as a subunit; for example, the T7-type phage is T7, T3, ϕ I, ϕ II, H, W31, gh-1, Y, A1122, or SP6, Studier, 95 Virology 70, 1979); the polymerase is non-discriminating for dideoxy nucleotide analogs; the polymerase is modified to have less than 50 units of exonuclease activity per mg of polymerase, more preferably less than 1 unit, even more preferably less than 0.1 unit, and most preferably has no detectable exonuclease activity; the polymerase is able to utilize primers of as short as 10 bases or preferably as short as 4 bases; the primer comprises four to forty nucleotide bases, and is single stranded DNA or RNA; the annealing step comprises heating the DNA molecule and the primer to above 65°C, preferably from 65°C to 100°C, and allowing the heated mixture to cool to below 65°C, preferably to 0°C to 30°C; the incubating step comprises a pulse and a chase step, wherein the pulse step comprises mixing the annealed mixture with all four different deoxynucleoside triphosphates and a processive DNA polymerase, wherein at least one of the deoxynucleoside triphosphates is labelled; most preferably the pulse step performed under conditions in which the polymerase does not exhibit its processivity and is for 30 seconds to 20 minutes at 0°C to 20°C or where at least one of the nucleotide triphosphates is limiting; and the chase step comprises adding one of the chain terminating agents to four separate aliquots of the mixture after the pulse step; preferably the chase step is for 1 to 60 minutes at 30°C to 50°C; the terminating agent is a dideoxynucleotide, or a limiting level of one deoxynucleoside triphosphate; one of the four deoxynucleotides is dITP or deazaguanosine; labelled primers are used so that no pulse step is required, preferably the label is radioactive or fluorescent; and the polymerase is unable to exhibit its processivity in a second environmental condition normally used in the pulse reaction of a DNA sequencing reaction.

In other aspects the invention features a) a method for producing blunt ended double-stranded DNA molecules from a linear DNA molecule having no 3' protruding termini, using a processive DNA polymerase free from exonuclease activity; b) a method of amplification of a DNA sequence comprising annealing a first and second primer to opposite strands of a double stranded DNA sequence and incubating the annealed

mixture with a processive DNA polymerase having less than 500 units of exonuclease activity per mg of polymerase, preferably less than 1 unit, wherein the first and second primers anneal to opposite strands of the DNA sequence; in preferred embodiments the primers have their 3' ends directed toward each other; and the method further comprises, after the incubation step, denaturing the resulting DNA, annealing the first and second primers to the resulting DNA and incubating the annealed mixture with the polymerase; preferably the cycle of denaturing, annealing and incubating is repeated from 10 to 40 times; c) a method for *in vitro* mutagenesis of cloned DNA fragments, comprising providing a cloned fragment and synthesizing a DNA strand using a processive DNA polymerase having less than 1 unit of exonuclease activity per mg of polymerase; d) a method of producing active T7-type DNA polymerase from cloned DNA fragments under the control of non-leaky promoters (see below) in the same cell comprising inducing expression of the genes only when the cells are in logarithmic growth phase, or stationary phase, and isolating the polymerase from the cell; preferably the cloned fragments are under the control of a promoter requiring T7 RNA polymerase for expression; e) a gene encoding a T7-type DNA polymerase, the gene being genetically modified to reduce the activity of naturally occurring exonuclease activity; most preferably a histidine (His) residue is modified, even more preferably His-123 of gene 5; f) the product of the gene encoding genetically modified polymerase; g) a method of purifying T7 DNA polymerase from cells comprising a vector from which the polymerase is expressed, comprising the steps of lysing the cells, and passing the polymerase over an ion-exchange column, over a DE52 DEAE column, a phosphocellulose column, and a hydroxyapatite column; preferably prior to the passing step the method comprises precipitating the polymerase with ammonium sulfate; the method further comprises the step of passing the polymerase over a Sephadex DEAE A50 column; and the ion-exchange column is a DE52 DEAE column; h) a method of inactivating exonuclease activity in a DNA polymerase solution comprising incubating the solution in a vessel containing oxygen, a reducing agent and a transition metal; i) a kit for DNA sequencing, comprising a processive DNA polymerase, defined as above, having less than 500 units of exonuclease activity per mg of polymerase, wherein the polymerase is able to exhibit its processivity in a first environmental condition, and preferably unable to exhibit its processivity in a second environmental condition, and a reagent necessary for the sequencing, selected from a chain terminating agent, and dITP; j) a method for labelling the 3' end of a DNA fragment comprising incubating the DNA fragment with a processive DNA polymerase having less than 500 units of exonuclease activity per mg of polymerase, and a labelled deoxynucleotide; k) a method for *in vitro* mutagenesis of a cloned DNA fragment comprising providing a primer and a template, the primer and the template having a specific mismatched base, and extending the primer with a processive DNA polymerase; and l) a method for *in vitro* mutagenesis of a cloned DNA fragment comprising providing the cloned fragment and synthesizing a DNA strand using a processive DNA polymerase, having less than 50 units of exonuclease activity, under conditions which cause misincorporation of a nucleotide base.

This invention provides a DNA polymerase which is processive, non-discriminating, and can utilize short primers. Further, the polymerase has no associated exonuclease activity. These are ideal properties for the above described methods, and in particular for DNA sequencing reactions, since the background level of radioactivity in the polyacrylamide gels is negligible, there are few or no artifactual bands, and the bands are sharp -- making the DNA sequence easy to read. Further, such a polymerase allows novel methods of sequencing long DNA fragments, as is described in detail below.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

45 Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

50 Figs. 1-3 are diagrammatic representations of the vectors pTrx-2, mGP1-1, and pGP5-5 respectively; Fig. 4 is a graphical representation of the selective oxidation of T7 DNA polymerase; Fig. 5 is a graphical representation of the ability of modified T7 polymerase to synthesize DNA in the presence of etheno-dATP; and
55 Fig. 6 is a diagrammatic representation of the enzymatic amplification of genomic DNA using modified T7 DNA polymerase.
Fig. 7, 8 and 9 are the nucleotide sequences of pTrx-2, a part of pGP5-5 and mGP1-2 respectively.
Fig. 10 is a diagrammatic representation of pGP5-6.

DNA Polymerase

In general the DNA polymerase of this invention is processive, has no associated exonuclease activity, does not discriminate against nucleotide analog incorporation, and can utilize small oligonucleotides (such as tetramers, hexamers and octamers) as specific primers. These properties will now be discussed in detail.

Processivity

By processivity is meant that the DNA polymerase is able to continuously incorporate many nucleotides using the same primer-template without dissociating from the template, under conditions normally used for DNA sequencing extension reactions. The degree of processivity varies with different polymerases: some incorporate only a few bases before dissociating (e.g. Klenow (about 15 bases), T4 DNA polymerase (about 10 bases), T5 DNA polymerase (about 180 bases) and reverse transcriptase (about 200 bases) (Das et al. J. Biol. Chem. 254:1227-1979; Bambara et al., J. Biol. Chem. 253:413, 1978) while others, such as those of the present invention, will remain bound for at least 500 bases and preferably at least 1,000 bases under suitable environmental conditions. Such environmental conditions include having adequate supplies of all four deoxynucleoside triphosphates and an incubation temperature from 10 °C-50 °C. Processivity is greatly enhanced in the presence of *E. coli* single stranded binding (ssb), protein.

With processive enzymes termination of a sequencing reaction will occur only at those bases which have incorporated a chain terminating agent, such as a dideoxynucleotide. If the DNA polymerase is non-processive, then artifactual bands will arise during sequencing reactions, at positions corresponding to the nucleotide where the polymerase dissociated. Frequent dissociation creates a background of bands at incorrect positions and obscures the true DNA sequence. This problem is partially corrected by incubating the reaction mixture for a long time (30-60 min) with a high concentration of substrates, which "chase" the artifactual bands up to a high molecular weight at the top of the gel, away from the region where the DNA sequence is read. This is not an ideal solution since a non-processive DNA polymerase has a high probability of dissociating from the template at regions of compact secondary structure, or hairpins. Reinitiation of primer elongation at these sites is inefficient and the usual result is the formation of bands at the same position for all four nucleotides, thus obscuring the DNA sequence.

Analog discrimination

The DNA polymerases of this invention do not discriminate significantly between dideoxy-nucleotide analogs and normal nucleotides. That is, the chance of incorporation of an analog is approximately the same as that of a normal nucleotide or at least incorporates the analog with at least 1/10 the efficiency that of a normal analog. The polymerases of this invention also do not discriminate significantly against some other analogs. This is important since, in addition to the four normal deoxynucleoside triphosphates (dGTP, dATP, dTTP and dCTP), sequencing reactions require the incorporation of other types of nucleotide derivatives such as: radioactively- or fluorescently-labelled nucleoside triphosphates, usually for labeling the synthesized strands with ³⁵S, ³²P, or other chemical agents. When a DNA polymerase does not discriminate against analogs the same probability will exist for the incorporation of an analog as for a normal nucleotide. For labelled nucleoside triphosphates this is important in order to efficiently label the synthesized DNA strands using a minimum of radioactivity. Further, lower levels of analogs are required with such enzymes, making the sequencing reaction cheaper than with a discriminating enzyme.

Discriminating polymerases show a different extent of discrimination when they are polymerizing in a processive mode versus when stalled, struggling to synthesize through a secondary structure impediment. At such impediments there will be a variability in the intensity of different radioactive bands on the gel, which may obscure the sequence.

Exonuclease Activity

The DNA polymerase of the invention has less than 50%, preferably less than 1%, and most preferably less than 0.1%, of the normal or naturally associated level of exonuclease activity (amount of activity per polymerase molecule). By normal or naturally associated level is meant the exonuclease activity of unmodified T7-type polymerase. Normally the associated activity is about 5,000 units of exonuclease activity per mg of polymerase, measured as described below by a modification of the procedure of Chase et al. (249 J. Biol. Chem. 4545, 1974). Exonucleases increase the fidelity of DNA synthesis by excising any newly synthesized bases which are incorrectly basepaired to the template. Such associated exonuclease

activities are detrimental to the quality of DNA sequencing reactions. They raise the minimal required concentration of nucleotide precursors which must be added to the reaction since, when the nucleotide concentration falls, the polymerase activity slows to a rate comparable with the exonuclease activity, resulting in no net DNA synthesis, or even degradation of the synthesized DNA.

5 More importantly, associated exonuclease activity will cause a DNA polymerase to idle at regions in the template with secondary structure impediments. When a polymerase approaches such a structure its rate of synthesis decreases as it struggles to pass. An associated exonuclease will excise the newly synthesized DNA when the polymerase stalls. As a consequence numerous cycles of synthesis and excision will occur. This may result in the polymerase eventually synthesizing past the hairpin (with no detriment to the quality
10 of the sequencing reaction); or the polymerase may dissociate from the synthesized strand (resulting in an artifactual band at the same position in all four sequencing reactions); or, a chain terminating agent may be incorporated at a high frequency and produce a wide variability in the intensity of different fragments in a sequencing gel. This happens because the frequency of incorporation of a chain terminating agent at any given site increases with the number of opportunities the polymerase has to incorporate the chain
15 terminating nucleotide, and so the DNA polymerase will incorporate a chain-terminating agent at a much higher frequency at sites of idling than at other sites.

An ideal sequencing reaction will produce bands of uniform intensity throughout the gel. This is essential for obtaining the optimal exposure of the X-ray film for every radioactive fragment. If there is variable intensity of radioactive bands, then fainter bands have a chance of going undetected. To obtain
20 uniform radioactive intensity of all fragments, the DNA polymerase should spend the same interval of time at each position on the DNA, showing no preference for either the addition or removal of nucleotides at any given site. This occurs if the DNA polymerase lacks any associated exonuclease, so that it will have only one opportunity to incorporate a chain terminating nucleotide at each position along the template.

25 Short primers

The DNA polymerase of the invention is able to utilize primers of 10 bases or less, as well as longer ones, most preferably of 4-20 bases. The ability to utilize short primers offers a number of important advantages to DNA sequencing. The shorter primers are cheaper to buy and easier to synthesize than the
30 usual 15-20-mer primers. They also anneal faster to complementary sites on a DNA template, thus making the sequencing reaction faster. Further, the ability to utilize small (e.g., six or seven base) oligonucleotide primers for DNA sequencing permits strategies not otherwise possible for sequencing long DNA fragments. For example, a kit containing 80 random hexamers could be generated, none of which are complementary to any sites in the cloning vector. Statistically, one of the 80 hexamer sequences will occur an average of
35 every 50 bases along the DNA fragment to be sequenced. The determination of a sequence of 3000 bases would require only five sequencing cycles. First, a "universal" primer (e.g., New England Biolabs #1211, sequence 5' GTAAAACGACGGCCAGT 3') would be used to sequence about 600 bases at one end of the insert. Using the results from this sequencing reaction, a new primer would be picked from the kit homologous to a region near the end of the determined sequence. In the second cycle, the sequence of the
40 next 600 bases would be determined using this primer. Repetition of this process five times would determine the complete sequence of the 3000 bases, without necessitating any subcloning, and without the chemical synthesis of any new oligonucleotide primers. The use of such short primers may be enhanced by including gene 2.5 and 4 protein of T7 in the sequencing reaction.

DNA polymerases of this invention, (i.e., having the above properties) include modified T7-type
45 polymerases. That is the DNA polymerase requires host thioredoxin as a sub-unit, and they are substantially identical to a modified T7 DNA polymerase or to equivalent enzymes isolated from related phage, such as T3, ϕ I, ϕ II, H, W31, gh-1, Y, A1122 and SP6. Each of these enzymes can be modified to have properties similar to those of the modified T7 enzyme. It is possible to isolate the enzyme from phage infected cells directly, but preferably the enzyme is isolated from cells which overproduce it. By substan-
50 tially identical is meant that the enzyme may have amino acid substitutions which do not affect the overall properties of the enzyme. One example of a particularly desirable amino acid substitution is one in which the natural enzyme is modified to remove any exonuclease activity. This modification may be performed at the genetic or chemical level (see below).

55 Cloning T7 polymerase

As an example of the invention we shall describe the cloning, overproduction, purification, modification and use of T7 DNA polymerase. This processive enzyme consists of two polypeptides tightly complexed in

a one to one stoichiometry. One is the phage T7-encoded gene 5 protein of 84,000 daltons (Modrich et al. 150 J. Biol. Chem. 5515, 1975), the other is the *E. coli* encoded thioredoxin, of 12,000 daltons (Tabor et al., J. Biol. Chem. 262:16, 216, 1987). The thioredoxin is an accessory protein and attaches the gene 5 protein (the non-processive actual DNA polymerase) to the primer template. The natural DNA polymerase has a very active 3' to 5' exonuclease associated with it. This activity makes the polymerase useless for DNA sequencing and must be inactivated or modified before the polymerase can be used. This is readily performed, as described below, either chemically, by local oxidation of the exonuclease domain, or genetically, by modifying the coding region of the polymerase gene encoding this activity.

10 pTrx-2

In order to clone the *trx*A (thioredoxin) gene of *E. coli* wild type *E. coli* DNA was partially cleaved with Sau3A and the fragments ligated to BamHI-cleaved T7 DNA isolated from strain T7 ST9 (Tabor et al., in Thioredoxin and Glutaredoxin Systems: Structure and Function (Holmgren et al., eds) pp. 285-300, Raven Press, NY; and Tabor et al., *supra*). The ligated DNA was transfected into *E. coli* *trx*A⁻ cells, the mixture plated onto *trx*A⁻ cells, and the resulting T7 plaques picked. Since T7 cannot grow without an active *E. coli* *trx*A gene only those phages containing the *trx*A gene could form plaques. The cloned *trx*A genes were located on a 470 base pair HincII fragment.

In order to overproduce thioredoxin a plasmid, pTrx-2, was as constructed. Briefly, the 470 base pair HincII fragment containing the *trx*A gene was isolated by standard procedure (Maniatis et al., Cloning: A Laboratory Manual, Cold Spring Harbor Labs., Cold Spring Harbor, N.Y.), and ligated to a derivative of pBR322 containing a Ptac promoter (ptac-12, Amann et al., 25 *Gene* 167, 1983). Referring to Fig. 2, ptac-12, containing β -lactamase and ColEI origin, was cut with PvuII, to yield a fragment of 2290 bp, which was then ligated to two tandem copies of *trx*A (HincII fragment) using commercially available linkers (SmaI-BamHI polylinker), to form pTrx-2. The complete nucleotide sequence of pTrx-2 is shown in Figure 7. Thioredoxin production is now under the control of the tac promoter, and thus can be specifically induced, e.g. by IPTG (isopropyl β -D-thiogalactoside).

pGP5-5 and mGP1-2

Some gene products of T7 are lethal when expressed in *E. coli*. An expression system was developed to facilitate cloning and expression of, lethal genes, based on the inducible expression of T7 RNA polymerase. Gene 5 protein is lethal in some *E. coli* strains and an example of such a system is described by Tabor et al. 82 *Proc. Nat. Acad. Sci.* 1074 (1985) where T7 gene 5 was placed under the control of the ϕ 10 promoter, and is only expressed when T7 RNA polymerase is present in the cell.

Briefly, pGP5-5 (Fig. 3) was constructed by standard procedures using synthetic BamHI linkers to join T7 fragment from 14306 (NdeI) to 16869 (AhaIII), containing gene 5, to the 560 bp fragment of T7 from 5667 (HincII) to 6166 (Fnu4HI) containing both the ϕ 1.1A and ϕ 1.1B promoters, which are recognized by T7 RNA polymerase, and the 3kb BamHI-HincII fragment of pACYC177 (Chang et al., 134 *J. Bacteriol.* 1141, 1978). The nucleotide sequence of the T7 inserts and linkers is shown in Fig. 8. In this plasmid gene 5 is only expressed when T7 RNA polymerase is provided in the cell.

Referring to Fig. 3, T7 RNA polymerase is provided on phage vector mGP1-2. This is similar to pGP1-2 (Tabor et al., *id.*) except that the fragment of T7 from 3133 (HaeIII) to 5840 (HinfI), containing T7 RNA polymerase was ligated, using linkers (BglII and Sall respectively), to BamHI-Sall cut M13 mp8, placing the polymerase gene under control of the lac promoter. The complete nucleotide sequence of mGP1-2 is shown in Fig. 9.

Since pGP5-5 and pTrx-2 have different origins of replication (respectively a P15A and a ColEI origin) they can be transformed into one cell simultaneously. pTrx-2 expresses large quantities of thioredoxin in the presence of IPTG. mGP1-2 can coexist in the same cell as these two plasmids and be used to regulate expression of T7-DNA polymerase from pGP5-5, simply by causing production of T7-RNA polymerase by inducing the lac promoter with, e.g., IPTG.

Overproduction of T7 DNA polymerase

There are several potential strategies for overproducing and reconstituting the two gene products of *trx*A and gene 5. The same cell strains and plasmids can be utilized for all the strategies. In the preferred strategy the two genes are co-overexpressed in the same cell. (This is because gene 5 is susceptible to proteases until thioredoxin is bound to it.) As described in detail below, one procedure is to place the two

genes separately on each of two compatible plasmids in the same cell. Alternatively, the two genes could be placed in tandem on the same plasmid. It is important that the T7-gene 5 is placed under the control of a non-leaky inducible promoter, such as $\phi 1.1A$, $\phi 1.1B$ and $\phi 10$ of T7, as the synthesis of even small quantities of the two polypeptides together is toxic in most *E. coli* cells. By non-leaky is meant that less than 500 molecules of the gene product are produced, per cell generation time, from the gene when the promoter, controlling the gene's expression, is not activated. Preferably the T7 RNA polymerase expression system is used although other expression systems which utilize inducible promoters could also be used. A leaky promoter, e.g., *plac*, allows more than 500 molecules of protein to be synthesized, even when not induced, thus cells containing lethal genes under the control of such a promoter grow poorly and are not suitable in this invention. It is of course possible to produce these products in cells where they are not lethal, for example, the *plac* promoter is suitable in such cells.

In a second strategy each gene can be cloned and overexpressed separately. Using this strategy, the cells containing the individually overproduced polypeptides are combined prior to preparing the extracts, at which point the two polypeptides form an active T7 DNA polymerase.

Example 1: Production of T7 DNA polymerase

E. coli strain 71.18 (Messing et al., Proc. Nat. Acad. Sci. 74:3642, 1977) is used for preparing stocks of mGP1-2. 71.18 is stored in 50% glycerol at -80°C . and is streaked on a standard minimal media agar plate. A single colony is grown overnight in 25 ml standard M9 media at 37°C , and a single plaque of mGP1-2 is obtained by titring the stock using freshly prepared 71.18 cells. The plaque is used to inoculate 10 ml 2X LB (2% Bacto-Tryptone, 1% yeast extract, 0.5% NaCl, 8mM NaOH) containing JM103 grown to an $A_{590} = 0.5$. This culture will provide the phage stock for preparing a large culture of mGP1-2. After 3-12 hours, the 10 ml culture is centrifuged, and the supernatant used to infect the large (2L) culture. For the large culture, 4 X 500 ml 2X LB is inoculated with 4 X 5 ml 71.18 cells grown in M9, and is shaken at 37°C . When the large culture of cells has grown to an $A_{590} = 1.0$ (approximately three hours), they are inoculated with 10 ml of supernatant containing the starter lysate of mGP1-2. The infected cells are then grown overnight at 37°C . The next day, the cells are removed by centrifugation, and the supernatant is ready to use for induction of K38/pGP5-5/pTrx-2 (see below). The supernatant can be stored at 4°C for approximately six months, at a titer $\sim 5 \times 10^{11}$ ϕ /ml. At this titer, 1 L of phage will infect 12 liters of cells at an $A_{590} = 5$ with a multiplicity of infection of 15. If the titer is low, the mGP1-2 phage can be concentrated from the supernatant by dissolving NaCl (60 gm/liter) and PEG-6000 (65 gm/liter) in the supernatant, allowing the mixture to settle at 0°C for 1-72 hours, and then centrifuging (7000 rpm for 20 min). The precipitate, which contains the mGP1-2 phage, is resuspended in approximately 1/20th of the original volume of M9 media.

K38/pGP5-5/pTrx-2 is the *E. coli* strain (genotype HfrC (λ)) containing the two compatible plasmids pGP5-5 and pTrx-2. pGP5-5 plasmid has a P15A origin of replication and expresses the kanamycin (Km) resistance gene. pTrx-2 has a ColEI origin of replication and expresses the ampicillin (Ap) resistance gene. The plasmids are introduced into K38 by standard procedures, selecting Km^R and Ap^R respectively. The cells K38/pGP5-5/pTrx-2 are stored in 50% glycerol at -80°C . Prior to use they are streaked on a plate containing 50 $\mu\text{g}/\text{ml}$ ampicillin and kanamycin, grown at 37°C overnight, and a single colony grown in 10 ml LB media containing 50 $\mu\text{g}/\text{ml}$ ampicillin and kanamycin, at 37°C for 4-6 hours. The 10 ml cell culture is used to inoculate 500 ml of LB media containing 50 $\mu\text{g}/\text{ml}$ ampicillin and kanamycin and shaken at 37°C overnight. The following day, the 500 ml culture is used to inoculate 12 liters of 2X LB-KPO₄ media (2% Bacto-Tryptone, 1% yeast extract, 0.5% NaCl, 20 mM KPO₄, 0.2% dextrose, and 0.2% casamino acids, pH 7.4), and grown with aeration in a fermentor at 37°C . When the cells reach an $A_{590} = 5.0$ (i.e. logarithmic or stationary phase cells), they are infected with mGP1-2 at a multiplicity of infection of 10, and IPTG is added (final concentration 0.5mM). The IPTG induces production of thioredoxin and the T7 RNA polymerase in mGP1-2, and thence induces production of the cloned DNA polymerase. The cells are grown for an additional 2.5 hours with stirring and aeration, and then harvested. The cell pellet is resuspended in 1.5 L 10% sucrose/20 mM Tris-HCl, pH 8.0/25 mM EDTA and re-spun. Finally, the cell pellet is resuspended in 200 ml 10% sucrose/20 mM Tris-HCl, pH 8/1.0 mM EDTA, and frozen in liquid N₂. From 12 liters of induced cells 70 gm of cell paste are obtained containing approximately 700 mg gene 5 protein and 100 mg thioredoxin.

K38/pTrx-2 (K38 containing pTrx-2 alone) overproduces thioredoxin, and it is added as a "booster" to extracts of K38/pGP5-5/pTrx-2 to insure that thioredoxin is in excess over gene 5 protein at the outset of the purification. The K38/pTrx-2 cells are stored in 50% glycerol at -80°C . Prior to use they are streaked on a plate containing 50 $\mu\text{g}/\text{ml}$ ampicillin, grown at 37°C for 24 hours, and a single colony grown at 37°C overnight in 25 ml LB media containing 50 $\mu\text{g}/\text{ml}$ ampicillin. The 25 ml culture is used to inoculate 2 L of 2X

LB media and shaken at 37°C. When the cells reach an $A_{590} = 3.0$, the *ptac* promoter, and thus thioredoxin production, is induced by the addition of IPTG (final concentration 0.5 mM). The cells are grown with shaking for an additional 12-16 hours at 37°C, harvested, resuspended in 600 ml 10% sucrose/20 mM Tris-HCl, pH 8.0/25 mM EDTA, and re-spun. Finally, the cells are resuspended in 40 ml 10% sucrose/20 mM Tris-HCl, pH 8.0/5 mM EDTA, and frozen in liquid N₂. From 2L of cells 16 gm of cell paste are obtained containing 150 mg of thioredoxin.

Assays for the polymerase involve the use of single-stranded calf thymus DNA (6mM) as a substrate. This is prepared immediately prior to use by denaturation of double-stranded calf thymus DNA with 50 mM NaOH at 20°C for 15 min., followed by neutralization with HCl. Any purified DNA can be used as a template for the polymerase assay, although preferably it will have a length greater than 1,000 bases.

The standard T7 DNA polymerase assay used is a modification of the procedure described by Grippo et al. (246 J. Biol. Chem. 6867, 1971). The standard reaction mix (200 µl final volume) contains 40 mM Tris/HCl pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, 100 nmol alkali-denatured calf thymus DNA, 0.3 mM dGTP, dATP, dCTP and [³H]dTTP (20 cpm/pM), 50 µg/ml BSA, and varying amounts of T7 DNA polymerase. Incubation is at 37°C (10°C-45°C) for 30 min (5 min-60 min). The reaction is stopped by the addition of 3 ml of cold (0°C) 1 N HCl-0.1 M pyrophosphate. Acid-insoluble radioactivity is determined by the procedure of Hinkle et al. (250 J. Biol. Chem. 5523, 1974). The DNA is precipitated on ice for 15 min (5 min-12 hr), then precipitated onto glass-fiber filters by filtration. The filters are washed five times with 4 ml of cold (0°C) 0.1M HCl-0.1M pyrophosphate, and twice with cold (0°C) 90% ethanol. After drying, the radioactivity on the filters is counted using a non-aqueous scintillation fluor.

One unit of polymerase activity catalyzes the incorporation of 10 nmol of total nucleotide into an acid-soluble form in 30 min at 37°C, under the conditions given above. Native T7 DNA polymerase and modified T7 DNA polymerase (see below) have the same specific polymerase activity $\pm 20\%$, which ranges between 5,000-20,000 units/mg for native and 5,000-50,000 units/mg for modified polymerase) depending upon the preparation, using the standard assay conditions stated above.

T7 DNA polymerase is purified from the above extracts by precipitation and chromatography techniques. An example of such a purification follows.

An extract of frozen cells (200 ml K38/pGP5-5/pTrx-2 and 40 ml K38/pTrx-2) are thawed at 0°C overnight. The cells are combined, and 5 ml of lysozyme (15 mg/ml) and 10 ml of NaCl (5M) are added. After 45 min at 0°C, the cells are placed in a 37°C water bath until their temperature reaches 20°C. The cells are then frozen in liquid N₂. An additional 50 ml of NaCl (5M) is added, and the cells are thawed in a 37°C water bath. After thawing, the cells are gently mixed at 0°C for 60 min. The lysate is centrifuged for one hr at 35,000 rpm in a Beckman 45Ti rotor. The supernatant (250 ml) is fraction I. It contains approximately 700 mg gene 5 protein and 250 mg of thioredoxin (a 2:1 ratio thioredoxin to gene 5 protein).

90 gm of ammonium sulphate is dissolved in fraction I (250 ml) and stirred for 60 min. The suspension is allowed to sit for 60 min, and the resulting precipitate collected by centrifugation at 8000 rpm for 60 min. The precipitate is redissolved in 300 ml of 20 mM Tris-HCl pH 7.5/5 mM 2-mercaptoethanol/0.1 mM EDTA/10% glycerol (Buffer A). This is fraction II.

A column of Whatman DE52 DEAE (12.6 cm² x 18 cm) is prepared and washed with Buffer A. Fraction II is dialyzed overnight against two changes of 1 L of Buffer A each until the conductivity of Fraction II has a conductivity equal to that of Buffer A containing 100 mM NaCl. Dialyzed Fraction II is applied to the column at a flow rate of 100 ml/hr, and washed with 400 ml of Buffer A containing 100 mM NaCl. Proteins are eluted with a 3.5 L gradient from 100 to 400 mM NaCl in Buffer A at a flow rate of 60 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 200 mM NaCl, are pooled. This is fraction III (190 ml).

A column of Whatman P11 phosphocellulose (12.6 cm² x 12 cm) is prepared and washed with 20 mM KPO₄ pH 7.4/5 mM 2-mercaptoethanol/0.1 mM EDTA/10 % glycerol (Buffer B). Fraction III is diluted 2-fold (380 ml) with Buffer B, then applied to the column at a flow rate of 60 ml/hr, and washed with 200 ml of Buffer B containing 100mM KCl. Proteins are eluted with a 1.8 L gradient from 100 to 400 mM KCl in Buffer B at a flow rate of 60 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 300 mM KCl, are pooled. This is fraction IV (370 ml).

A column of DEAE-Sephadex A-50 (4.9 cm² x 15 cm) is prepared and washed with 20 mM Tris-HCl 7.0/0.1 mM dithiothreitol/0.1 mM EDTA/10% glycerol (Buffer C). Fraction IV is dialyzed against two changes of 1 L Buffer C to a final conductivity equal to that of Buffer C containing 100 mM NaCl. Dialyzed fraction IV is applied to the column at a flow rate of 40 ml/hr, and washed with 150 ml of Buffer C containing 100 mM NaCl. Proteins are eluted with a 1 L gradient from 100 to 300 mM NaCl in Buffer C at a flow rate of 40 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 210 mM NaCl, are pooled. This is fraction V (120 ml).

A column of BioRad HTP hydroxylapatite (4.9 cm² x 15 cm) is prepared and washed with 20 mM KPO₄.

pH 7.4/10 mM 2-mercaptoethanol/2 mM Na citrate/10% glycerol (Buffer D). Fraction V is dialyzed against two changes of 500 ml Buffer D each. Dialyzed fraction V is applied to the column at a flow rate of 30 ml/hr, and washed with 100 ml of Buffer D. Proteins are eluted with a 900 ml gradient from 0 to 180 mM KPO_4 , pH 7.4 in Buffer D at a flow rate of 30 ml/hr. Fractions containing T7 DNA polymerase, which elutes at 50 mM KPO_4 , are pooled. This is fraction VI (130 ml). It contains 270 mg of homogeneous T7 DNA polymerase.

Fraction VI is dialyzed versus 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/0.1 mM EDTA/50% glycerol. This is concentrated fraction VI (~65 ml, 4 mg/ml), and is stored at -20°C .

The isolated T7 polymerase has exonuclease activity associated with it. As stated above this must be inactivated. An example of inactivation by chemical modification follows.

Concentrated fraction VI is dialyzed overnight against 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/10% glycerol to remove the EDTA present in the storage buffer. After dialysis, the concentration is adjusted to 2 mg/ml with 20 mM KPO_4 pH 7.4/0.1 mM dithiothreitol/10% glycerol, and 30 ml (2mg/ml) aliquots are placed in 50 ml polypropylene tubes. (At 2 mg/ml, the molar concentration of T7 DNA polymerase is 22 μM .)

Dithiothreitol (DTT) and ferrous ammonium sulfate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$) are prepared fresh immediately before use, and added to a 30 ml aliquot of T7 DNA polymerase, to concentrations of 5 mM DTT (0.6 ml of a 250 mM stock) and 20 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 ml of a 1 mM stock). During modification the molar concentrations of T7 DNA polymerase and iron are each approximately 20 μM , while DTT is in 250X molar excess.

The modification is carried out at 0°C under a saturated oxygen atmosphere as follows. The reaction mixture is placed on ice within a dessicator, the dessicator is purged of air by evacuation and subsequently filled with 100% oxygen. This cycle is repeated three times. The reaction can be performed in air (20% oxygen), but occurs at one third the rate.

The time course of loss of exonuclease activity is shown in Fig. 4. ^3H -labeled double-stranded DNA (6 cpm/pmol) was prepared from bacteriophage T7 as described by Richardson (15 J. Molec. Biol. 49, 1966). ^3H -labeled single-stranded T7 DNA was prepared immediately prior to use by denaturation of double-stranded ^3H -labeled T7 DNA with 50 mM NaOH at 20°C for 15 min, followed by neutralization with HCl. The standard exonuclease assay used is a modification of the procedure described by Chase et al. (supra). The standard reaction mixture (100 μl final volume) contained 40 mM Tris/HCl pH 7.5, 10 mM MgCl_2 , 10 mM dithiothreitol, 60 nmol ^3H -labeled single-stranded T7 DNA (6 cpm/pmol), and varying amounts of T7 DNA polymerase. ^3H -labeled double-stranded T7 DNA can also be used as a substrate. Also, any uniformly radioactively labeled DNA, single- or double-stranded, can be used for the assay. Also, 3' end labeled single- or double-stranded DNA can be used for the assay. After incubation at 37°C for 15 min, the reaction is stopped by the addition of 30 μl of BSA (10mg/ml) and 25 μl of TCA (100% w/v). The assay can be run at 10°C - 45°C for 1-60 min. The DNA is precipitated on ice for 15 min (1 min - 12 hr), then centrifuged at 12,000 g for 30 min (5 min - 3 hr). 100 μl of the supernatant is used to determine the acid-soluble radioactivity by adding it to 400 μl water and 5 ml of aqueous scintillation cocktail.

One unit of exonuclease activity catalyzes the acid solubilization of 10 nmol of total nucleotide in 30 min under the conditions of the assay. Native T7 DNA polymerase has a specific exonuclease activity of 5000 units/mg, using the standard assay conditions stated above. The specific exonuclease activity of the modified T7 DNA polymerase depends upon the extent of chemical modification, but ideally is at least 10-100-fold lower than that of native T7 DNA polymerase, or 500 to 50 or less units/mg using the standard assay conditions stated above. When double stranded substrate is used the exonuclease activity is about 7-fold higher.

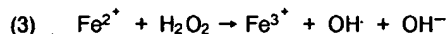
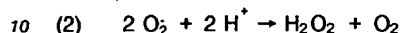
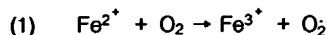
Under the conditions outlined, the exonuclease activity decays exponentially, with a half-life of decay of eight hours. Once per day the reaction vessel is mixed to distribute the soluble oxygen, otherwise the reaction will proceed more rapidly at the surface where the concentration of oxygen is higher. Once per day 2.5 mM DTT (0.3 ml of a fresh 250 mM stock to a 30 ml reaction) is added to replenish the oxidized DTT.

After eight hours, the exonuclease activity of T7 DNA polymerase has been reduced 50%, with negligible loss of polymerase activity. The 50% loss may be the result of the complete inactivation of exonuclease activity of half the polymerase molecules, rather than a general reduction of the rate of exonuclease activity in all the molecules. Thus, after an eight hour reaction all the molecules have normal polymerase activity, half the molecules have normal exonuclease activity, while the other half have $<0.1\%$ of their original exonuclease activity.

When 50% of the molecules are modified (an eight hour reaction), the enzyme is suitable, although suboptimal, for DNA sequencing. For more optimum quality of DNA sequencing, the reaction is allowed to proceed to greater than 99% modification (having less than 50 units of exonuclease activity), which requires four days.

After four days, the reaction mixture is dialyzed against 2 changes of 250 ml of 20 mM KPO₄ pH 7.4/0.1 mM dithiothreitol/0.1 mM EDTA/50% glycerol to remove the iron. The modified T7 DNA polymerase (~4 mg/ml) is stored at -20 °C.

The reaction mechanism for chemical modification of T7 DNA polymerase depends upon reactive oxygen species generated by the presence of reduced transition metals such as Fe²⁺ and oxygen. A possible reaction mechanism for the generation of hydroxyl radicals is outlined below:



In equation 1, oxidation of the reduced metal ion yields superoxide radical, O₂^{·-}. The superoxide radical can undergo a dismutation reaction, producing hydrogen peroxide (equation 2). Finally, hydrogen peroxide can react with reduced metal ions to form hydroxyl radicals, OH[·] (the Fenton reaction, equation 3). The oxidized metal ion is recycled to the reduced form by reducing agents such as dithiothreitol (DTT).

These reactive oxygen species probably inactivate proteins by irreversibly chemically altering specific amino acid residues. Such damage is observed in SDS-PAGE of fragments of gene 5 produced by CNBr or trypsin. Some fragments disappear, high molecular weight cross linking occurs, and some fragments are broken into two smaller fragments.

As previously mentioned, oxygen, a reducing agent (e.g. DTT, 2-mercaptoethanol) and a transition metal (e.g. iron) are essential elements of the modification reaction. The reaction occurs in air, but is stimulated three-fold by use of 100% oxygen. The reaction will occur slowly in the absence of added transition metals due to the presence of trace quantities of transition metals (1-2 μM) in most buffer preparations.

As expected, inhibitors of the modification reaction include anaerobic conditions (e.g., N₂) and metal chelators (e.g. EDTA, citrate, nitrilotriacetate). In addition, the enzymes catalase and superoxide dismutase may inhibit the reaction, consistent with the essential role of reactive oxygen species in the generation of modified T7 DNA polymerase.

As an alternative procedure, it is possible to genetically mutate the T7 gene 5 to specifically inactivate the exonuclease domain of the protein. The T7 gene 5 protein purified from such mutants is ideal for use in DNA sequencing without the need to chemically inactivate the exonuclease by oxidation and without the secondary damage that inevitably occurs to the protein during chemical modification.

Genetically modified T7 DNA polymerase can be isolated by randomly mutagenizing the gene 5 and then screening for those mutants that have lost exonuclease activity, without loss of polymerase activity. Mutagenesis is performed as follows. Single-stranded DNA containing gene 5 (e.g., cloned in pEMBL-8, a plasmid containing an origin for single stranded DNA replication) under the control of a T7 RNA polymerase promoter is prepared by standard procedure, and treated with two different chemical mutagens: hydrazine, which will mutate C's and T's, and formic acid, which will mutate G's and A's. Myers et al. 229 Science 242, 1985. The DNA is mutagenized at a dose which results in an average of one base being altered per plasmid molecule. The single-stranded mutagenized plasmids are then primed with a universal 17-mer primer (see above), and used as templates to synthesize the opposite strands. The synthesized strands contain randomly incorporated bases at positions corresponding to the mutated bases in the templates. The double-stranded mutagenized DNA is then used to transform the strain K38/pGP1-2, which is strain K38 containing the plasmid pGP1-2 (Tabor et al., supra). Upon heat induction this strain expresses T7 RNA polymerase. The transformed cells are plated at 30 °C, with approximately 200 colonies per plate.

Screening for cells having T7 DNA polymerase lacking exonuclease activity is based upon the following finding. The 3' to 5' exonuclease of DNA polymerases serves a proofreading function. When bases are misincorporated, the exonuclease will remove the newly incorporated base which is recognized as "abnormal". This is the case for the analog of dATP, etheno-dATP, which is readily incorporated by T7 DNA polymerase in place of dATP. However, in the presence of the 3' to 5' exonuclease of T7 DNA polymerase, it is excised as rapidly as it is incorporated, resulting in no net DNA synthesis. As shown in figure 6, using the alternating copolymer poly d(AT) as a template, native T7 DNA polymerase catalyzes extensive DNA synthesis only in the presence of dATP, and not etheno-dATP. In contrast, modified T7 DNA polymerase, because of its lack of an associated exonuclease, stably incorporates etheno-dATP into DNA at a rate comparable to dATP. Thus, using poly d(AT) as a template, and dTTP and etheno-dATP as precursors, native T7 DNA polymerase is unable to synthesize DNA from this template, while T7 DNA

polymerase which has lost its exonuclease activity will be able to use this template to synthesize DNA.

The procedure for lysing and screening large number of colonies is described in Raetz (72 Proc. Nat. Acad. Sci. 2274, 1975). Briefly, the K38/pGP1-2 cells transformed with the mutagenized gene 5-containing plasmids are transferred from the petri dish, where they are present at approximately 200 colonies per plate, to a piece of filter paper ("replica plating"). The filter paper discs are then placed at 42°C for 60 min to induce the T7 RNA polymerase, which in turn expresses the gene 5 protein. Thioredoxin is constitutively produced from the chromosomal gene. Lysozyme is added to the filter paper to lyse the cells. After a freeze thaw step to ensure cell lysis, the filter paper discs are incubated with poly d(AT), [$\alpha^{32}\text{P}$]dTTP and etheno-dATP at 37°C for 60 min. The filter paper discs are then washed with acid to remove the unincorporated [^{32}P]dATP. DNA will precipitate on the filter paper in acid, while nucleotides will be soluble. The washed filter paper is then used to expose X-ray film. Colonies which have induced an active T7 DNA polymerase which is deficient in its exonuclease will have incorporated acid-insoluble ^{32}P , and will be visible by autoradiography. Colonies expressing native T7 DNA polymerase, or expressing a T7 DNA polymerase defective in polymerase activity, will not appear on the autoradiograph.

Colonies which appear positive are recovered from the master petri dish containing the original colonies. Cells containing each potential positive clone will be induced on a larger scale (one liter) and T7 DNA polymerase purified from each preparation to ascertain the levels of exonuclease associated with each mutant. Those low in exonuclease are appropriate for DNA sequencing.

Directed mutagenesis may also be used to isolate genetic mutants in the exonuclease domain of the T7 gene 5 protein. The following is an example of this procedure.

T7 DNA polymerase with reduced exonuclease activity (modified T7 DNA polymerase) can also be distinguished from native T7 DNA polymerase by its ability to synthesize through regions of secondary structure. Thus, with modified DNA polymerase, DNA synthesis from a labeled primer on a template having secondary structure will result in significantly longer extensions, compared to unmodified or native DNA polymerase. This assay provides a basis for screening for the conversion of small percentages of DNA polymerase molecules to a modified form.

The above assay was used to screen for altered T7 DNA polymerase after treatment with a number of chemical reagents. Three reactions resulted in conversion of the enzyme to a modified form. The first is treatment with iron and a reducing agent, as described above. The other two involve treatment of the enzyme with photooxidizing dyes, Rose Bengal and methylene blue, in the presence of light. The dyes must be titrated carefully, and even under optimum conditions the specificity of inactivation of exonuclease activity over polymerase activity is low, compared to the high specificity of the iron-induced oxidation. Since these dyes are quite specific for modification of histidine residues, this result strongly implicates histidine residues as an essential species in the exonuclease active site.

There are 23 histidine residues in T7 gene 5 protein. Eight of these residues lie in the amino half of the protein, in the region where, based on the homology with the large fragment of *E. coli* DNA polymerase I, the exonuclease domain may be located (Ollis et al. Nature 313, 818, 1984). As described below, seven of the eight histidine residues were mutated individually by synthesis of appropriate oligonucleotides, which were then incorporated into gene 5. These correspond to mutants 1, and 6-10 in table 1.

The mutations were constructed by first cloning the T7 gene 5 from pGP5-3 (Tabor et al., J. Biol. Chem. 262, 1987) into the *Sma*I and *Hind*III sites of the vector M13 mp18, to give mGP5-2. (The vector used and the source of gene 5 are not critical in this procedure.) Single-stranded mGP5-2 DNA was prepared from a strain that incorporates deoxyuracil in place of deoxythymidine (Kunkel, Proc. Natl. Acad. Sci. USA 82, 488, 1985). This procedure provides a strong selection for survival of only the synthesized strand (that containing the mutation) when transfected into wild-type *E. coli*, since the strand containing uracil will be preferentially degraded.

Mutant oligonucleotides, 15-20 bases in length, were synthesized by standard procedures. Each oligonucleotide was annealed to the template, extended using native T7 DNA polymerase, and ligated using T4 DNA ligase. Covalently closed circular molecules were isolated by agarose gel electrophoresis, run in the presence of 0.5 $\mu\text{g/ml}$ ethidium bromide. The resulting purified molecules were then used to transform *E. coli* 71.18. DNA from the resulting places was isolated and the relevant region sequenced to confirm each mutation.

The following summarizes the oligonucleotides used to generate genetic mutants in the gene 5 exonuclease. The mutations created are underlined. Amino acid and base pair numbers are taken from Dunn et al., 166 J. Molec. Biol. 477, 1983. The relevant wild type sequences of the region of gene 5 mutated are also shown.

Wild type sequence:

109 (aa) 122 123
 5 Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser His Ala Leu Glu
 CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG TCT CAC GCT TTG GAG
 14677 (17 bp)

10

Mutation 1: His 123 → Ser 123

15 Primer used: 5' CGC TTT GGA TCC TCC GCT TTG 3'

Mutant sequence:

123
 20 Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser Ser Ala Leu Glu
 CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGA TCC TCC GCT TTG GAG

20

Mutation 2: Deletion of Ser 122 and His 123

Primer used: 5' GGA AAA CGC TTT GGC GCG TTG GAG GCG 3'

25

Δ
 6 base deletion

Mutant sequence:

122 123
 30 Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ala Leu Glu
 CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGC --- --- GCG TTG GAG

30

35

40

45

50

55

Mutation 3: Ser 122, His 123 → Ala 122, Glu 123

Primer used: 5' CGC TTT GGG GCT GAG GCT TTG G 3'

Mutant sequence:

122 123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ala Glu Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG GCT GAG GCT TTG GAG

Mutation 4: Lys 118, Arg 119 → Glu 118, Glu 119

Primer used: 5' 5' G CCC GGG GAA GAG TTT GGG TCT CAC GC 3'

Mutant sequence:

118 119
Leu Leu Arg Ser Gly Lys Leu Pro Gly Glu Glu Phe Gly Ser His Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGG GAA GAG TTT GGG TCT CAC GCT TTG GAG

Mutation 5: Arg 111, Ser 112, Lys 114 → Glu 111, Ala 112, Glu 114

Primer used: 5' G GGT CTT CTG GAA GCC GGC GAG TTG CCC GG 3'

Mutant sequence:

111 112 114
Leu Leu Glu Ala Gly Glu Leu Pro Gly Lys Arg Phe Gly Ser His Ala Leu
Glu
CTT CTG GAA GCC GGC GAG TTG CCC GGA AAA CGC TTT GGG TCT CAC GCT TTG GAG

Mutation 6: His 59, His 62 → Ser 59, Ser 62

Primer used: 5' ATT GTG TTC TCC AAC GGA TCC AAG TAT GAC G 3'

Wild-type sequence:

aa: 55 59 62
Leu Ile Val Phe His Asn Gly His Lys Tyr Asp Val
CTT ATT GTG TTC CAC AAC GGT CAC AAG TAT GAC GTT
T7 bp: 14515

Mutant sequence:

59 62
Leu Ile Val Phe Ser Asn Gly Ser Lys Tyr Asp Val
CTT ATT GTG TTC TCC AAC GGA TCC AAG TAT GAC GTT

Mutation 7: His 82 → Ser 82

Primer used: 5' GAG TTC ~~TCC~~ CTT CCT CG 3'

Wild-type sequence:

aa: 77 82
Leu Asn Arg Glu Phe His Leu Pro Arg Glu Asn
TTG AAC CGA GAG TTC CAC CTT CCT CGT GAG AAC
T7 bp: 14581

Mutant sequence:

82
Leu Asn Arg Glu Phe ~~Ser~~ Leu Pro Arg Glu Asn
TTG AAC CGA GAG TTC ~~TCC~~ CTT CCT CGT GAG AAC

Mutation 8: Arg 96, His 99 → Leu 96, Ser 99

Primer used: 5' ~~CTG~~ TTG ATT ~~TCT~~ TCC AAC CTC 3'

Wild-type sequence:

aa: 93 96 99
Val Leu Ser Arg Leu Ile His Ser Asn Leu Lys Asp Thr Asp
GTG TTG TCA CGT TTG ATT CAT TCC AAC CTC AAG GAC ACC GAT
T7 bp: 14629

Mutant sequence:

96 99
Val Leu Ser ~~Leu~~ Leu Ile ~~Ser~~ Ser Asn Leu Lys Asp Thr Asp
GTG TTG TCA ~~CTG~~ TTG ATT ~~TCT~~ TCC AAC CTC AAG GAC ACC GAT

Mutation 9: His 190 → Ser 190

Primer used: 5' CT GAC AAA ~~TCT~~ TAC TTC CCT 3'

Wild-type sequence:

aa: 185 190
Leu Leu Ser Asp Lys His Tyr Phe Pro Pro Glu
CTA CTC TCT GAC AAA CAT TAC TTC CCT CCT GAG
T7 bp: 14905

Mutant sequence:

190
Leu Leu Ser Asp Lys ~~Ser~~ Tyr Phe Pro Pro Glu
CTA CTC TCT GAC AAA ~~TCT~~ TAC TTC CCT CCT GAG

Mutation 10: His 218 → Ser 218

Primer used: 5' GAC ATT GAA ~~TCT~~ CGT GCT GC 3'

Wild-type sequence:

aa: 214 218
Val Asp Ile Glu His Arg Ala Ala Trp Leu Leu
GTT GAC ATT GAA CAT CGT GCT GCA TGG CTG CTC

T7 bp: 14992

Mutant sequence:

218
Val Asp Ile Glu ~~Ser~~ Arg Ala Ala Trp Leu Leu
GTT GAC ATT GAA ~~TCT~~ CGT GCT GCA TGG CTG CTC

Mutation 11: Deletion of amino acids 118 to 123

Primer used: 5' C GGC AAG TTG CCC GGG GCT TTG GAG GCG TGG G 3'

Δ
18 base deletion

Wild-type sequence:

109 (aa) 118 122 123 126
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser His Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG TCT CAC GGT TTG GAG
14677 (T7 bp)

Mutant sequence:

117 124
Leu Leu Arg Ser Gly Lys Leu Pro Gly.....(6 amino acids).....Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGG.....(18 bases).....GCT TTG GAG

Mutation 12: His 123 → Glu 123

Primer used: 5' GGG TCT GAG GCT TTG G 3'

Mutant sequence:

123
Leu Leu Arg Ser Gly Lys Leu Pro Gly Lys Arg Phe Gly Ser Glu Ala Leu Glu
CTT CTG CGT TCC GGC AAG TTG CCC GGA AAA CGC TTT GGG TCT GAG GCT TTG GAG

Mutation 13: (Arg 131, Lys 136, Lys 140, Lys 144, Arg 145 → Glu 131, Glu 136, Glu 140, Glu 144, Glu 145)

Primer used: 5' GGT TAT GAG ~~CTC~~ GGC GAG ATG GAG GGT GAA TAC GAA GAC GAC TTT GAG GAA A
CTT GAA G 3'

Wild-type sequence:

129(aa) 131 136 140 144 145
Gly Tyr Arg Leu Gly Glu Met Lys Gly Glu Tyr Lys Asp Asp Phe Lys Arg Met Leu Glu Glu
GGT TAT CGC TTA GGC GAG ATG AAG GGT GAA TAC AAA GAC GAC TTT AAG CGT ATG CTT GAA G
14737 (17 bp)

Mutant sequence:

129(aa) 131 136 140 144 145
Gly Tyr Glu Leu Gly Glu Met Glu Gly Glu Tyr Glu Asp Asp Phe Glu Glu Met Leu Glu Glu
GGT TAT GAG TAC GGC GAG ATG GAG GGT GAA TAC GAA GAC GAC TTT GAG GAA ATG CTT GAA G
14737 (T7 bp)

Each mutant gene 5 protein was produced by infection of the mutant phage into K38/pGP1-2, as follows. The cells were grown at 30 °C to an $A_{590} = 1.0$. The temperature was shifted to 42 °C for 30 min., to induce T7 RNA polymerase. IPTG was added to 0.5 mM, and a lysate of each phage was added at a moi = 10. Infected cells were grown at 37 °C for 90 min. The cells were then harvested and extracts prepared by standard procedures for T7 gene 5 protein.

Extracts were partially purified by passage over a phosphocellulose and DEAE A-50 column, and assayed by measuring the polymerase and exonuclease activities directly, as described above. The results are shown in Table 1.

Table 1
SUMMARY OF EXONUCLEASE AND POLYMERASE
ACTIVITIES OF T7 GENE 5 MUTANTS

| | <u>Mutant</u> | <u>Exonuclease activity, %</u> | <u>Polymerase activity, %</u> |
|----|---|--------------------------------|-------------------------------|
| | [Wild-type] | [100] ^a | [100] ^b |
| 45 | Mutant 1 (His 123 → Ser 123) | 10-25 | >90 |
| 50 | Mutant 2 (Δ Ser 122, His 123) | 0.2-0.4 | >90 |
| | Mutant 3 (Ser 122, His 123 → Ala 122, Glu 123) | <2 | >90 |

Table 1
SUMMARY OF EXONUCLEASE AND POLYMERASE
ACTIVITIES OF T7 GENE 5 MUTANTS

| | <u>Mutant</u> | <u>Exonuclease activity, %</u> | <u>Polymerase activity, %</u> |
|----|---|------------------------------------|-----------------------------------|
| 5 | Mutant 4 (Lys 118, Arg 119 → Glu 118, Glu 119) | <30 | >90 |
| 10 | Mutant 5 (Arg 111, Ser 112, Lys 114 → Glu 111, Ala 112, Glu 114) | >75 | >90 |
| 15 | Mutant 6 (His 59, His 62 → Ser 59, Ser 62) | >75 | >90 |
| | Mutant 7 (His 82 → Ser 82) | >75 | >90 |
| 20 | Mutant 8 (Arg 96, His 99 → Leu 96, Ser 99) | >75 | >90 |
| | Mutant 9 (His 190 → Ser 190) | >75 | >90 |
| 25 | Mutant 10 (His 218 → Ser 218) | >75 | >90 |
| 30 | Mutant 11 (Δ Lys 118, Arg 119, Phe 120, Gly 121, Ser 122, His 123) | <0.02 | >90 |
| | Mutant 12 (His 123 → Glu 123) | <30 | >90 |
| 35 | Mutant 13 (Arg 131, Lys 136, Lys 140, Lys 144, Arg 145 → Glu 131, Glu 136, Glu 140, Glu 144, Glu 145) | <30 | >90 |

40 a. Exonuclease activity was measured on single stranded [³H]T7
DNA. 100% exonuclease activity corresponds to 5,000 units/mg.

b. Polymerase activity was measured using single-stranded calf thymus
DNA. 100% polymerase activity corresponds to 8,000 units/mg.

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Of the seven histidines tested, only one (His 123: mutant 1) has the enzymatic activities characteristic of modified T7 DNA polymerase. T7 gene 5 protein was purified from this mutant using DEAE-cellulose, phosphocellulose, DEAE-Sephadex and hydroxylapatite chromatography. While the polymerase activity was nearly normal (>90% the level of the native enzyme), the exonuclease activity was reduced 4 to 10-fold.

A variant of this mutant was constructed in which both His 123 and Ser 122 were deleted. The gene 5 protein purified from this mutant has a 200-500 fold lower exonuclease activity, again with retention of >90% of the polymerase activity.

These data strongly suggest that His 123 lies in the active site of the exonuclease domain of T7 gene 5 protein. Furthermore, it is likely that the His 123 is in fact the residue being modified by the oxidation involving iron, oxygen and a reducing agent, since such oxidation has been shown to modify histidine residues in other proteins (Levine, J. Biol. Chem. 258: 11823, 1983; and Hodgson et al. Biochemistry 14: 5294, 1975). The level of residual exonuclease in mutant 11 is comparable to the levels obtainable by

chemical modification.

Although mutations at His residues are described, mutations at nearby sites or even at distant sites may also produce mutant enzymes suitable in this invention, e.g., lys and arg (mutants 4 and 15). Similarly, although mutations in some His residues have little effect on exonuclease activity that does not necessarily indicate that mutations near these residues will not affect exonuclease activity. Mutations which are especially effective include those having deletions of 2 or more amino acids, preferably 6-8, for example, near the His-123 region. Other mutations should reduce exonuclease activity further, or completely.

As an example of the use of these mutant strains the following is illustrative. A pGP5-6 (mutation 11)-containing strain has been deposited with the ATCC (see below). The strain is grown as described above and induced as described in Taber et al. J. Biol. Chem. 262:16212 (1987). K38/pTrx-2 cells may be added to increase the yield of genetically modified T7 DNA polymerase.

The above noted deposited strain also contains plasmid pGP1-2 which expresses T7 RNA polymerase. This plasmid is described in Tabor et al., Proc. Nat. Acad. Sci. USA 82:1074, 1985 and was deposited with the ATCC on March 22, 1985 and assigned the number 40,175.

Referring to Fig. 10, pGP5-6 includes the following segments:

1. EcoRI-SacI-SmaI-BamHI polylinker sequence from M13 mp10 (21bp).
2. T7 bp 14309 to 16747, that contains the T7 gene 5, with the following modifications:
T7 bp 14703 is changed from an A to a G, creating a SmaI site.
T7 bp 14304 to 14321 inclusive are deleted (18 bp).
3. SalI-PstI-HindIII polylinker sequence from M13 mp 10 (15 bp)
4. pBR322 bp 29 (HindIII site) to pBR322 bp 375 (BamHI site).
5. T7 bp 22855 to T7 bp 22927, that contains the T7 RNA Polymerase promoter ϕ 10, with BamHI linkers inserted at each end (82 bp).
6. pBR322 bp 375 (BamHI site) to pBR322 bp 4361 (EcoRI site).

DNA Sequencing Using Modified T7-type DNA Polymerase

DNA synthesis reactions using modified T7-type DNA polymerase result in chain-terminated fragments of uniform radioactive intensity, throughout the range of several bases to thousands of bases in length. There is virtually no background due to terminations at sites independent of chain terminating agent incorporation (i.e. at pause sites or secondary structure impediments).

Sequencing reactions using modified T7-type DNA polymerase consist of a pulse and chase. By pulse is meant that a short labelled DNA fragment is synthesized; by chase is meant that the short fragment is lengthened until a chain terminating agent is incorporated. The rationale for each step differs from conventional DNA sequencing reactions. In the pulse, the reaction is incubated at 0°C-37°C for 0.5-4 min in the presence of high levels of three nucleotide triphosphates (e.g., dGTP, dCTP and dTTP) and limiting levels of one other labelled, carrier-free, nucleotide triphosphate, e.g., [³⁵S] dATP. Under these conditions the modified polymerase is unable to exhibit its processive character, and a population of radioactive fragments will be synthesized ranging in size from a few bases to several hundred bases. The purpose of the pulse is to radioactively label each primer, incorporating maximal radioactivity while using minimal levels of radioactive nucleotides. In this example, two conditions in the pulse reaction (low temperature, e.g., from 0-20°C, and limiting levels of dATP, e.g., from 0.1μM to 1μM) prevent the modified T7-type DNA polymerase from exhibiting its processive character. Other essential environmental components of the mixture will have similar effects, e.g., limiting more than one nucleotide triphosphate or increasing the ionic strength of the reaction. If the primer is already labelled (e.g., by kinasing) no pulse step is required.

In the chase, the reaction is incubated at 45°C for 1-30 min in the presence of high levels (50-500μM) of all four deoxynucleoside triphosphates and limiting levels (1-50μM) of any one of the four chain terminating agents, e.g., dideoxynucleoside triphosphates, such that DNA synthesis is terminated after an average of 50-600 bases. The purpose of the chase is to extend each radioactively labeled primer under conditions of processive DNA synthesis, terminating each extension exclusively at correct sites in four separate reactions using each of the four dideoxynucleoside triphosphates. Two conditions of the chase (high temperature, e.g., from 30-50°C) and high levels (above 50μM) of all four deoxynucleoside triphosphates allow the modified T7-type DNA polymerase to exhibit its processive character for tens of thousands of bases; thus the same polymerase molecule will synthesize from the primer-template until a dideoxynucleotide is incorporated. At a chase temperature of 45°C synthesis occurs at >700 nucleotides/sec. Thus, for sequencing reactions the chase is complete in less than a second. ssb increases processivity, for example, when using dITP, or when using low temperatures or high ionic strength, or low levels of triphosphates throughout the sequencing reaction.

Either [$\alpha^{35}\text{S}$]dATP, [$\alpha^{32}\text{P}$]dATP or fluorescently labelled nucleotides can be used in the DNA sequencing reactions with modified T7-type DNA polymerase. If the fluorescent analog is at the 5' end of the primer, then no pulse step is required.

Two components determine the average extensions of the synthesis reactions. First is the length of time of the pulse reaction. Since the pulse is done in the absence of chain terminating agents, the longer the pulse reaction time, the longer the primer extensions. At 0°C the polymerase extensions average 10 nucleotides/sec. Second is the ratio of deoxyribonucleoside triphosphates to chain terminating agents in the chase reaction. A modified T7-type DNA polymerase does not discriminate against the incorporation of these analogs, thus the average length of extension in the chase is four times the ratio of the deoxynucleoside triphosphate concentration to the chain terminating agent concentration in the chase reaction. Thus, in order to shorten the average size of the extensions, the pulse time is shortened, e.g., to 30 sec. and/or the ratio of chain terminating agent to deoxynucleoside triphosphate concentration is raised in the chase reaction. This can be done either by raising the concentration of the chain terminating agent or lowering the concentration of deoxynucleoside triphosphate. To increase the average length of the extensions, the pulse time is increased, e.g., to 3-4 min, and/or the concentration of chain terminating agent is lowered (e.g., from 20 μM to 2 μM) in the chase reaction.

Example 2: DNA sequencing using modified T7 DNA polymerase

The following is an example of a sequencing protocol using dideoxy nucleotides as terminating agents. 9 μl of single-stranded M13 DNA (mGP1-2, prepared by standard procedures) at 0.7 mM concentration is mixed with 1 μl of complementary sequencing primer (standard universal 17-mer, 0.5 pmole primer / μl) and 2.5 μl 5X annealing buffer (200 mM Tris-HCl, pH 7.5, 50 mM MgCl_2) heated to 65°C for 3 min, and slow cooled to room temperature over 30 min. In the pulse reaction, 12.5 μl of the above annealed mix was mixed with 1 μl dithiothreitol 0.1 M, 2 μl of 3 dNTPs (dGTP, dCTP, dTTP) 3 mM each (P.L. Biochemicals, in TE), 2.5 μl [$\alpha^{35}\text{S}$]dATP, (1500 Ci/mmol, New England Nuclear) and 1 μl of modified T7 DNA polymerase described in Example 1 (0.4 mg/ml, 2500 units/ml, i.e. 0.4 μg , 2.5 units) and incubated at 0°C, for 2 min, after vortexing and centrifuging in a microfuge for 1 sec. The time of incubation can vary from 30 sec to 20 min and temperature can vary from 0°C to 37°C. Longer times are used for determining sequences distant from the primer.

4.5 μl aliquots of the above pulse reaction are added to each of four tubes containing the chase mixes, preheated to 45°C. The four tubes, labeled G, A, T, C, each contain trace amounts of either dideoxy (dd) G, A, T, or C (P-L Biochemicals). The specific chase solutions are given below. Each tube contains 1.5 μl dATP 1mM, 0.5 μl 5X annealing buffer (200 mM Tris-HCl, pH 7.5, 50mM MgCl_2), and 1.0 μl ddNTP 100 μM (where ddNTP corresponds to ddG,A,T or C in the respective tubes). Each chase reaction is incubated at 45°C (or 30°C-50°C) for 10 min, and then 6 μl of stop solution (90% formamide, 10mM EDTA, 0.1% xlenecyanol) is added to each tube, and the tube placed on ice. The chase times can vary from 1-30 min.

The sequencing reactions are run on standard, 6% polyacrylamide sequencing gel in 7M urea, at 30 Watts for 6 hours. Prior to running on a gel the reactions are heated to 75°C for 2 min. The gel is fixed in 10% acetic acid, 10% methanol, dried on a gel dryer, and exposed to Kodak OM1 high-contrast autoradiography film overnight.

Example 3: DNA sequencing using limiting concentrations of dNTPs

In this example DNA sequence analysis of mGP1-2 DNA is performed using limiting levels of all four deoxyribonucleoside triphosphates in the pulse reaction. This method has a number of advantages over the protocol in example 2. First, the pulse reaction runs to completion, whereas in the previous protocol it was necessary to interrupt a time course. As a consequence the reactions are easier to run. Second, with this method it is easier to control the extent of the elongations in the pulse, and so the efficiency of labeling of sequences near the primer (the first 50 bases) is increased approximately 10-fold.

7 μl of 0.75 mM single-stranded M13 DNA (mGP1-2) was mixed with 1 μl of complementary sequencing primer (17-mer, 0.5 pmole primer/ μl) and 2 μl 5X annealing buffer (200 mM Tris-HCl pH 7.5, 50 mM MgCl_2 , 250 mM NaCl) heated at 65°C for 2 min, and slowly cooled to room temperature over 30 min. In the pulse reaction 10 μl of the above annealed mix was mixed with 1 μl dithiothreitol 0.1 M, 2 μl of 3 dNTPs (dGTP, dCTP, dTTP) 1.5 μM each, 0.5 μl [$\alpha^{35}\text{S}$]dATP, (about 10 μM , 1500 Ci/mmol, New England Nuclear) and 2 μl modified T7 DNA polymerase (0.1 mg/ml, 1000 units/ml, i.e. , 0.2 μg , 2 units) and incubated at 37°C for 5 min. (The temperature and time of incubation can be varied from 20°C-45°C and 1-60 min., respectively.)

3.5 μ l aliquots of the above pulse reaction were added to each of four tubes containing the chase mixes, which were preheated to 37°C. The four tubes, labeled G, A, T, C, each contain trace amounts of either dideoxy G, A, T, C. The specific chase solutions are given below. Each tube contains 0.5 μ l 5X annealing buffer (200 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 250 mM NaCl), 1 μ l 4dNTPs (dGTP, dATP, dTTP, dCTP) 200 μ M each, and 1.0 μ l ddNTP 20 μ M. Each chase reaction is incubated at 37°C for 5 min (or 20°C-45°C and 1-60 min respectively), and then 4 μ l of a stop solution (95% formamide, 20 mM EDTA, 0.05% xylene-cyanol) added to each tube, and the tube placed on ice prior to running on a standard polyacrylamide sequencing gel as described above.

10 Example 4: Replacement of dGTP with dITP for DNA sequencing

In order to sequence through regions of compression in DNA, i.e., regions having compact secondary structure, it is common to use dITP (Mills et al., 76 Proc. Natl. Acad. Sci. 2232, 1979) or deazaguanosine triphosphate (deaza GTP, Mizusawa et al., 14 Nuc. Acid Res. 1319, 1986). We have found that both analogs function well with T7-type polymerases, especially with dITP in the presence of *ssb*. Preferably these reactions are performed with the above described genetically modified T7 polymerase, or the chase reaction is for 1-2 min., and/or at 20°C to reduce exonuclease degradation.

Modified T7 DNA polymerase efficiently utilizes dITP or deaza-GTP in place of dGTP. dITP is substituted for dGTP in both the pulse and chase mixes at a concentration two to five times that at which dGTP is used. In the ddG chase mix ddGTP is still used (not ddITP).

The chase reactions using dITP are sensitive to the residual low levels (about 0.01 units) of exonuclease activity. To avoid this problem, the chase reaction times should not exceed 5 min when dITP is used. It is recommended that the four dITP reactions be run in conjunction with, rather than to the exclusion of, the four reactions using dGTP. If both dGTP and dITP are routinely used, the number of required mixes can be minimized by: (1) Leaving dGTP and dITP out of the chase mixes, which means that the four chase mixes can be used for both dGTP and dITP chase reactions. (2) Adding a high concentration of dGTP or dITP (2 μ l at 0.5 mM and 1-2.5 mM respectively) to the appropriate pulse mix. The two pulse mixes then each contain a low concentration of dCTP, dTTP and [α -³⁵S]dATP, and a high concentration of either dGTP or dITP. This modification does not usually adversely effect the quality of the sequencing reactions, and reduces the required number of pulse and chase mixes to run reactions using both dGTP and dITP to six.

The sequencing reaction is as for example 3, except that two of the pulse mixes contain a) 3 dNTP mix for dGTP: 1.5 μ M dCTP, dTTP, and 1 mM dGTP and b) 3 dNTP mix for dITP: 1.5 μ M dCTP, dTTP, and 2 mM dITP. In the chase reaction dGTP is removed from the chase mixes (i.e. the chase mixes contain 30 μ M dATP, dTTP and dCTP, and one of the four dideoxynucleotides at 8 μ M), and the chase time using dITP does not exceed 5 min.

Deposits

Strains K38/pGP5-5/pTrx-2, K38/pTrx-2 and M13 mGP1-2 have been deposited with the ATCC and assigned numbers 67,287, 67,286, and 40,303 respectively. These deposits were made on January 13, 1987. Strain K38/pGP1-2/pGP5-6 was deposited with the ATCC. On December 4, 1987, and assigned the number 67571.

Applicants' and their assignees acknowledge their responsibility to replace these cultures should they die before the end of the term of a patent issued hereon, 5 years after the last request for a culture, or 30 years, whichever is the longer, and its responsibility to notify the depository of the issuance of such a patent, at which time the deposits will be made irrevocably available to the public. Until that time the deposits will be made irrevocably available to the Commissioner of Patents under the terms of 37 CFR Section 1-14 and 35 USC Section 112.

50 Other Embodiments

Other embodiments are within the following claims.

Other uses of the modified DNA polymerases of this invention, which take advantage of their processivity, and lack of exonuclease activity, include the direct enzymatic amplification of genomic DNA sequences. This has been described, for other polymerases, by Saiki et al., 230 Science 1350, 1985; and Scharf, 233 Science 1076, 1986.

Referring to Fig. 6, enzymatic amplification of a specific DNA region entails the use of two primers which anneal to opposite strands of a double stranded DNA sequence in the region of interest, with their 3'

ends directed toward one another (see dark arrows). The actual procedure involves multiple (10-40, preferably 16-20) cycles of denaturation, annealing, and DNA synthesis. Using this procedure it is possible to amplify a specific region of human genomic DNA over 200,000 times. As a result the specific gene fragment represents about one part in five, rather than the initial one part in a million. This greatly facilitates both the cloning and the direct analysis of genomic DNA. For diagnostic uses, it can speed up the analysis from several weeks to 1-2 days.

Unlike Klenow fragment, where the amplification process is limited to fragments under two hundred bases in length, modified T7-type DNA polymerases should (preferably in conjunction with E. coli DNA binding protein, or ssb, to prevent "snapback formation of single stranded DNA) permit the amplification of DNA fragments thousands of bases in length.

The modified T7-type DNA polymerases are also suitable in standard reaction mixtures: for a) filling in 5' protruding termini of DNA fragments generated by restriction enzyme cleavage; in order to, for example, produce blunt-ended double stranded DNA from a linear DNA molecule having a single stranded region with no 3' protruding termini; b) for labeling the 3' termini of restriction fragments, for mapping mRNA start sites by S1 nuclease analysis, or sequencing DNA using the Maxam and Gilbert chemical modification procedure; and c) for in vitro mutagenesis of cloned DNA fragments. For example, a chemically synthesized primer which contains specific mismatched bases is hybridized to a DNA template, and then extended by the modified T7-type DNA polymerase. In this way the mutation becomes permanently incorporated into the synthesized strand. It is advantageous for the polymerase to synthesize from the primer through the entire length of the DNA. This is most efficiently done using a processive DNA polymerase. Alternatively mutagenesis is performed by misincorporation during DNA synthesis (see above). This application is used to mutagenize specific regions of cloned DNA fragments. It is important that the enzyme used lack exonuclease activity. By standard reaction mixture is meant a buffered solution containing the polymerase and any necessary deoxynucleosides, or other compounds.

Claims

1. A method for determining the nucleotide base sequence of a DNA molecule, comprising the steps of:
 - providing said DNA molecule annealed with a primer molecule able to hybridize to said DNA molecule,
 - incubating the annealed mixture with a DNA polymerase, except reverse transcriptase, in the presence of four different deoxynucleoside triphosphates and a chain terminating agent which terminates DNA synthesis at a specific nucleotide base, wherein the concentration of all four deoxynucleoside triphosphates at the start of said incubating is sufficient to allow DNA synthesis to continue until terminated by said agent, and
 - separating the DNA products of said incubating reaction according to their size, whereby at least a part of the nucleotide base sequence of said DNA molecule can be determined.
2. A method according to claim 1 wherein prior to said incubating the primer is extended with a DNA polymerase, except reverse transcriptase, in the presence of one or more deoxynucleoside triphosphates, at least one of said deoxynucleoside triphosphates being labelled.
3. A method according to claims 1 or 2 wherein separate portions of said annealed mixture are incubated in at least four vessels, each vessel containing four different deoxynucleotide triphosphates and a chain terminating agent which chain terminating agent terminates DNA synthesis at a different specific nucleotide base in each of said vessels.
4. A method according to claim 1 wherein said annealed mixture is incubated with said DNA polymerase and four different deoxynucleoside triphosphates in a single vessel.
5. A method according to claim 1 wherein the primer is labelled.
6. A method according to of claims 1 to 5 wherein said DNA polymerase is a processive DNA polymerase.
7. A method according to claim 6 wherein said DNA polymerase is a T7-type DNA polymerase.
8. A method according to claim 7 wherein said T7-type DNA polymerase is T7 DNA polymerase.

9. A method according to any of claims 1 to 8 wherein said polymerase has a level of exonuclease activity which is sufficiently low to permit the nucleotide base sequence of the DNA molecule to be determined.
- 5 10. A method according to claim 9 wherein said DNA polymerase has less than 10% of the exonuclease activity of the naturally associated level of exonuclease activity of said polymerase.
11. A method according to claim 10 wherein said DNA polymerase has less than 1% of the exonuclease activity of the naturally associated level of exonuclease activity of said polymerase.
- 10 12. A method according to claim 6 wherein said DNA polymerase remains bound to said DNA molecule for at least 500 bases before dissociating.
13. A method according to claim 12 wherein said polymerase remains bound to said DNA molecule for at least 1,000 bases before dissociating.
- 15 14. A method according to claim 8 wherein said polymerase is substantially the same as that in cells infected with a T7-type phage.
- 20 15. A method according to claim 14 wherein said T7-type phage is T7, T3, ϕ I, ϕ II, W31, gh-1, Y, A1122 or Sp6.
16. A method according to any of claims 1 to 15 wherein said polymerase is non-discriminating for dideoxy nucleotide analogs.
- 25 17. A method according to any of claims 1 to 16 wherein said DNA polymerase has less than 500 units of exonuclease activity per mg of DNA polymerase.
18. A method according to claim 17 wherein said DNA polymerase has less than 50 units of exonuclease activity per mg of DNA polymerase.
- 30 19. A method according to claim 18 wherein said DNA polymerase has less than 1 unit of exonuclease activity per mg of DNA polymerase.
- 35 20. A method according to claim 19 wherein said DNA polymerase has no detectable exonuclease activity.
21. A method according to any of claims 1 to 20 wherein said DNA polymerase is able to utilize primers of 10 base pairs or more.
- 40 22. A method according to claim 21 wherein said polymerase is able to utilize primers of 4 base pairs or more.
23. A method according to any of claims 1 to 22 wherein said terminating agent is a dideoxynucleotide

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FIGURE 1

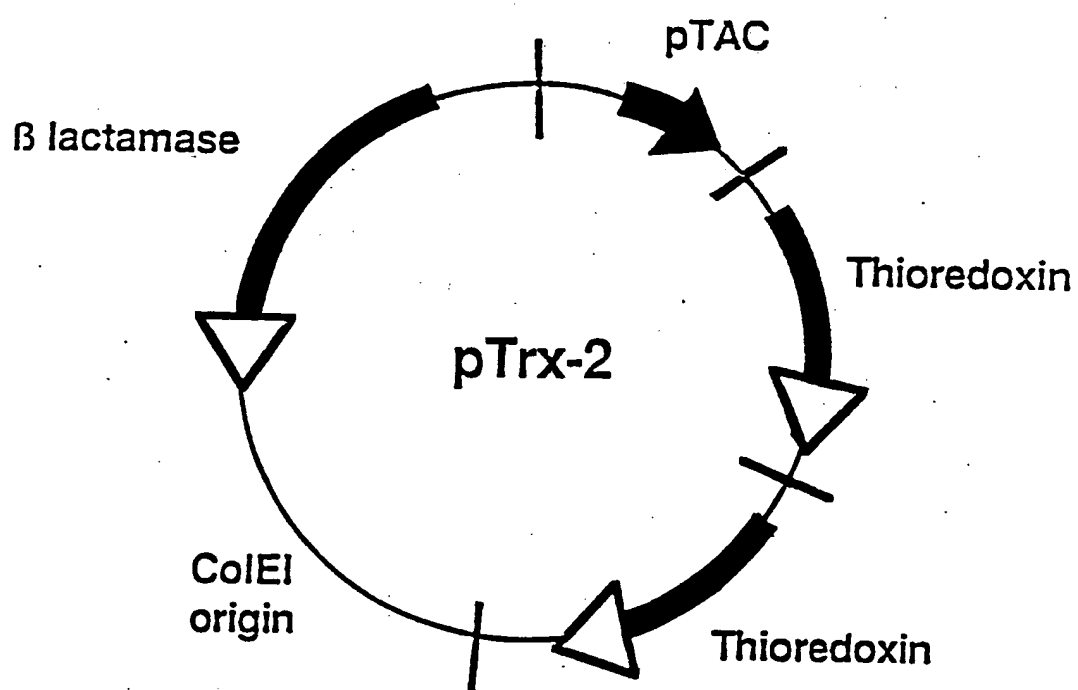


FIGURE 2

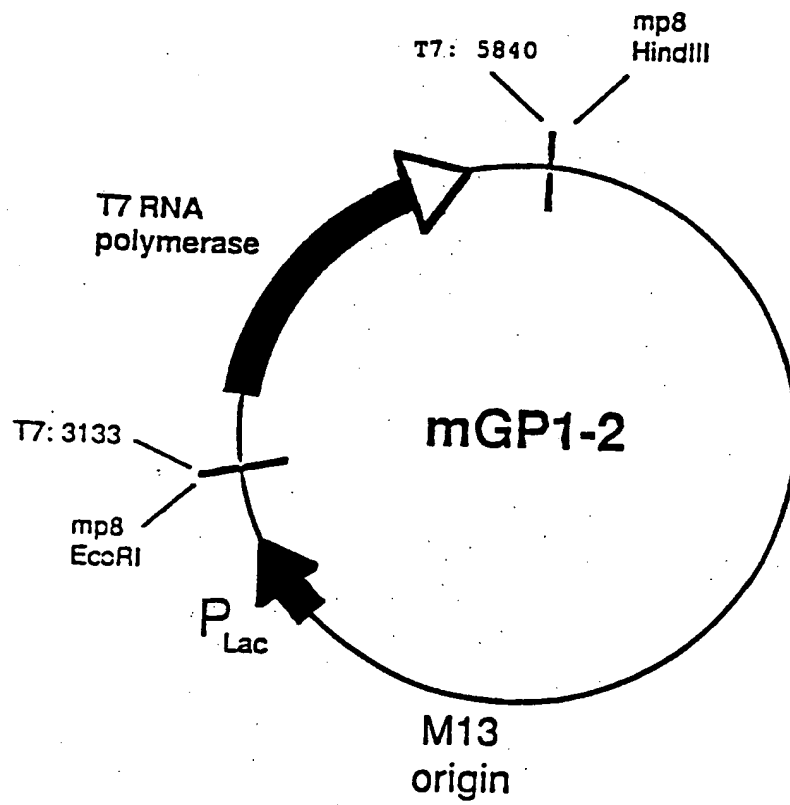


FIGURE 3

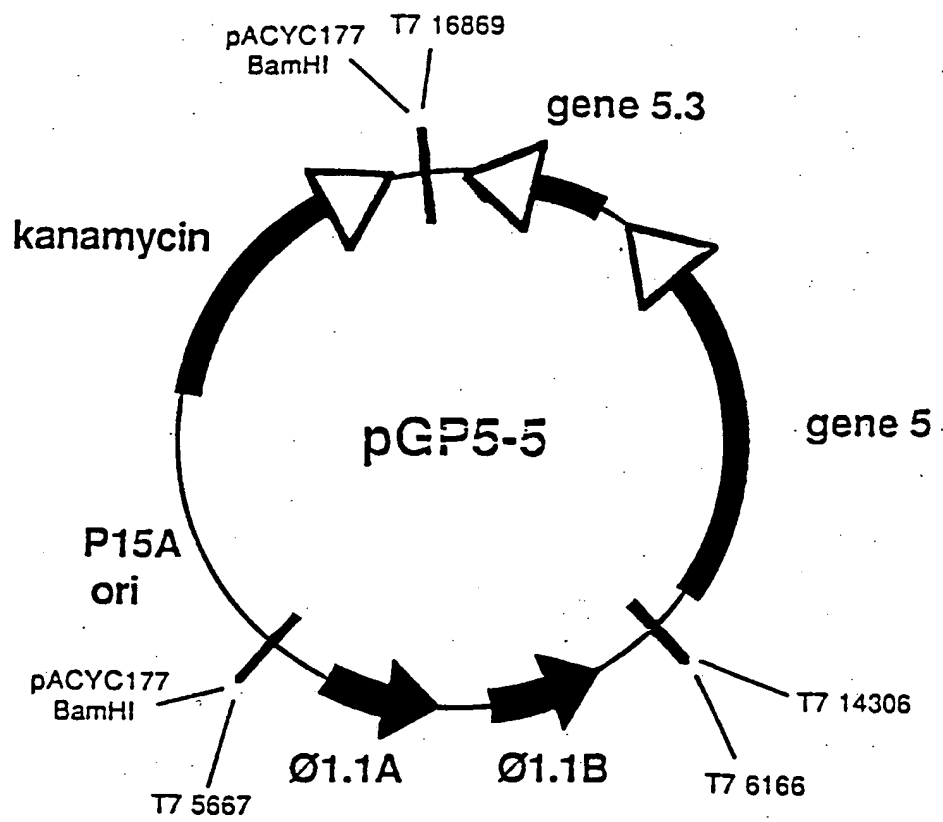


FIGURE 4

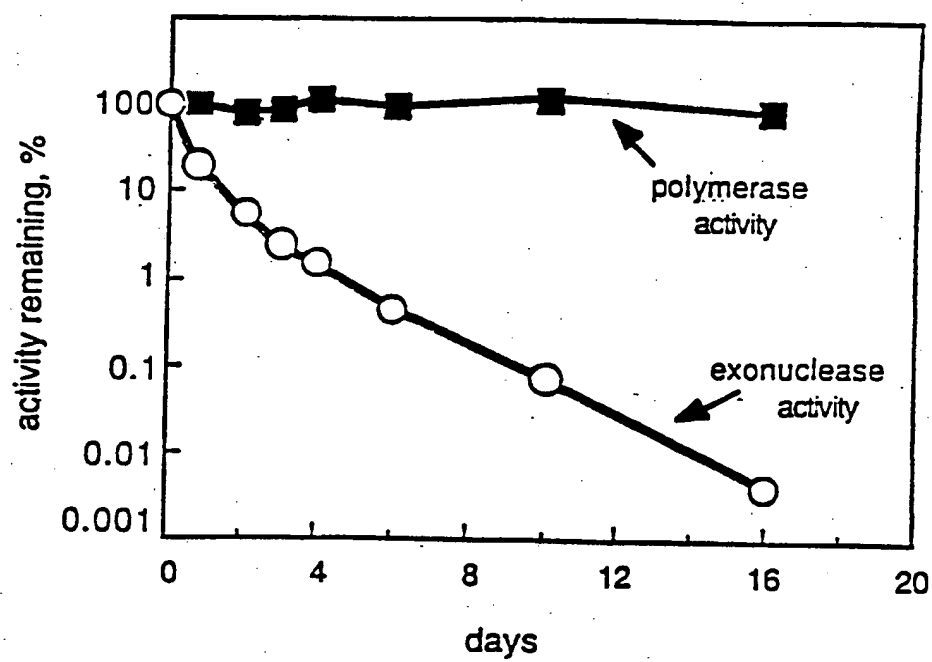


FIGURE 5

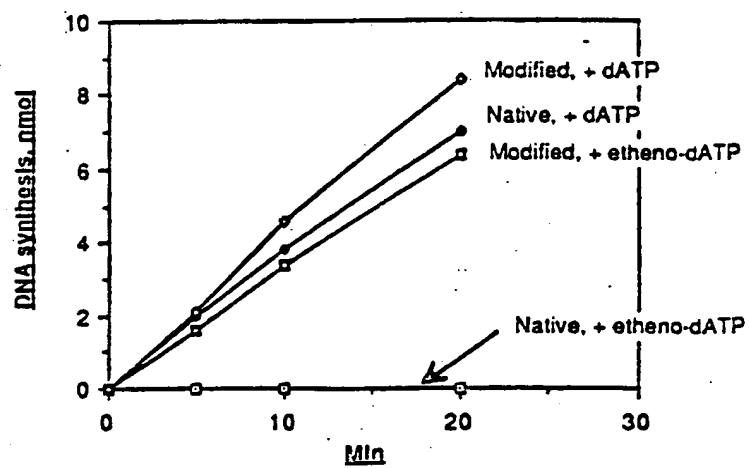
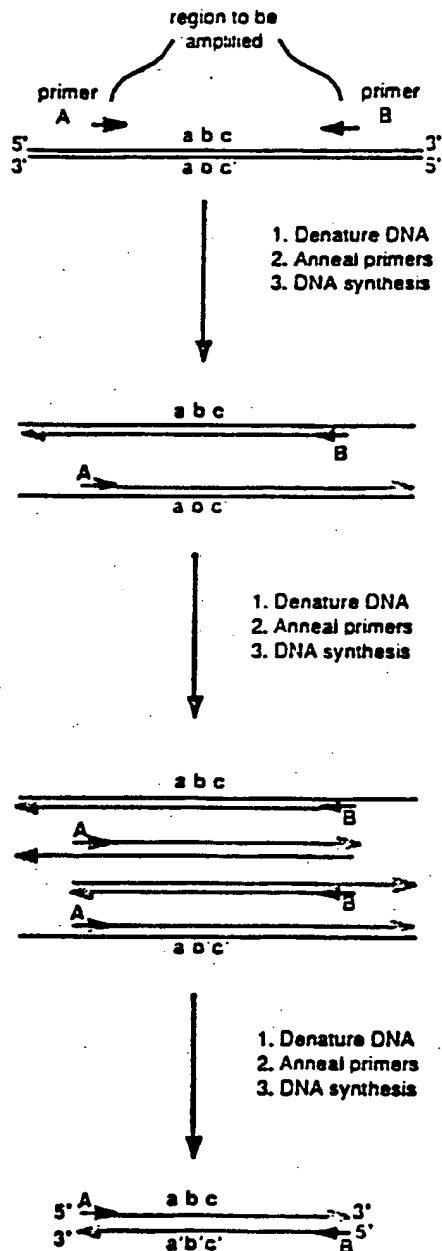


FIGURE 6



Previously synthesized strands now serve as templates.

Repeat cycle of denaturation, annealing, and DNA synthesis 16 more times.

Region between two primers amplified 2^{18} , or 1,000,000 fold.

FIGURE 7

| | | | | |
|------------|------------|------------|------------|-------------|
| 10 | 20 | 30 | 40 | 50 |
| TTCTTCTCAT | GTTTGACAGC | TTATCATCGA | CTGCACGGTG | CACCAATGCT |
| 60 | 70 | 80 | 90 | 100 |
| TCTGGCGTCA | GGCAGCCATC | GGAAGCTGTG | GTATGGCTGT | GCAGGTCGTA |
| 110 | 120 | 130 | 140 | 150 |
| AATCACTGCA | TAATTCGTGT | CGCTCAAGGC | GCACTCCCGT | TCTGGATAAT |
| 160 | 170 | 180 | 190 | 200 |
| GTTTTTTTGG | CCGACATCAT | AACGGTTCTG | GCAAATATTC | TGAAATGAGC |
| 210 | 220 | 230 | 240 | 250 |
| TGTTGACAAT | TAATCATCGG | CTCGTATAAT | GTGTGGAATT | GTGAGCGGAT |
| 260 | 270 | 280 | 290 | 300 |
| AACAATTTCA | CACAGGAAAC | AGGGGATCCG | TCAACCTTTA | GTTGGTTAAT |
| 310 | 320 | 330 | 340 | 350 |
| GTTACACCAA | CAACGAAACC | AACACGCCAG | GCTTATTCCT | GTGGAGTTAT |
| 360 | 370 | 380 | 390 | 400 |
| ATATGAGCGA | TAAAATTATT | CACCTGACTG | ACGACAGTTT | TGACACGGAT |
| 410 | 420 | 430 | 440 | 450 |
| GTAATCAAAG | CGGACGGGGC | GATCCTCGTC | GATTTCTGGG | CAGAGTGGTG |
| 460 | 470 | 480 | 490 | 500 |
| CGGTCCGTGC | AAGATGATCG | CCCCGATTCT | GGATGAAATC | GCTGACGRAAT |

FIGURE 7 (continued)

| | | | | |
|-------------|------------|-------------|------------|------------|
| 510 | 520 | 530 | 540 | 550 |
| ATCAGGGCAA | ACTGACCGTT | GCAAACTGA | ACATCGATCA | AAACCCTGGT |
| 560 | 570 | 580 | 590 | 600 |
| ACTGCGCCGA | AATATGGCAT | CCGTGGTATC | CCGACTCTGC | TGCTGTCAA |
| 610 | 620 | 630 | 640 | 650 |
| AAACGGTGAA | GTGGCGGCAA | CCAAAGTGGG | TGCACTGTCT | AAAGGTCAGT |
| 660 | 670 | 680 | 690 | 700 |
| TGAAAGAGTT | CCTCGACGCT | AACCTGGCGT | AAGGGAATTT | CATGTTCCGG |
| 710 | 720 | 730 | 740 | 750 |
| TGCCCCGTGC | CTAAAACTG | GACGCCCCGC | GTGAGTCATG | CTAACTTAGT |
| 760 | 770 | 780 | 790 | 800 |
| GTTGACGGAT | CCCCGGGGAT | CCGTCAACCT | TTAGTTGGTT | AATGTTACAC |
| 810 | 820 | 830 | 840 | 850 |
| CAACAACGAA | ACCAACACGC | CAGGCTTATT | CCTGTGGAGT | TATATATGAG |
| 860 | 870 | 880 | 890 | 900 |
| CGATAAAATT | ATTCACCTGA | CTGACGACAG | TTTTGACACG | GATGACTCA |
| 910 | 920 | 930 | 940 | 950 |
| AAGCGGACGG | GGCGATCCTC | GTCGATTCT | GGGCAGAGTG | GTGCGGTCCG |
| 960 | 970 | 980 | 990 | 1000 |
| TGCAAGATGA | TGCCCCGAT | TCTGGATGAA | ATCGCTGACG | AATATCAGGG |
| 1010 | 1020 | 1030 | 1040 | 1050 |
| CAAAC TGACC | GTTGCAAAAC | TGAACATCGA | TCAAACCCT | GGTACTGCGC |
| 1060 | 1070 | 1080 | 1090 | 1100 |
| CGAAATATGG | CATCCGTGGT | ATCCC GACTC | TGCTGCTGTT | CAAAAACGGT |
| 1110 | 1120 | 1130 | 1140 | 1150 |
| GAAGTGGCGG | CAACCAAAGT | GGGTGCACTG | TCTAAAGGTC | AGTTGAAAGA |
| 1160 | 1170 | 1180 | 1190 | 1200 |
| GTTCCCTCGAC | GCTAACCTGG | CGTAAGGGAA | TTTCATGTTC | GGGTGCCCCG |
| 1210 | 1220 | 1230 | 1240 | 1250 |
| TCGCTAAAAA | CTGGACGCCC | GGCGTGAGTC | ATGCTAACTT | AGTGTGACG |
| 1260 | 1270 | 1280 | 1290 | 1300 |
| GATCCCCCTG | CCTCGCGCGT | TTCGGTGATG | ACGGTGAAAA | CCTCTGACAC |
| 1310 | 1320 | 1330 | 1340 | 1350 |
| ATGCAGCTCC | CGGAGACGGT | CACAGCTTGT | CTGTAAGCGG | ATGCCGGGAG |
| 1360 | 1370 | 1380 | 1390 | 1400 |
| CAGACAAGCC | CGTCAGGGCG | CGTCAGCGGG | TGTTGGCGGG | TGTCGGGGCG |
| 1410 | 1420 | 1430 | 1440 | 1450 |
| CAGCCATGAC | CCAGTCACGT | AGCGATAGCG | GAGTGTATAC | TGGCTTAACT |
| 1460 | 1470 | 1480 | 1490 | 1500 |
| ATGCGGCATC | AGAGCAGATT | GTA CTGAGAG | TGCACCATAT | GCGGTGTGAA |
| 1510 | 1520 | 1530 | 1540 | 1550 |
| ATACCGCACA | GATGCGTAAG | GAGAAAATAC | CGCATCAGGC | GCTCTTCCGC |
| 1560 | 1570 | 1580 | 1590 | 1600 |
| TTCCTCGCTC | ACTGACTCGC | TGCGCTCGGT | CGTTCGGCTG | CGGCGAGCGG |
| 1610 | 1620 | 1630 | 1640 | 1650 |
| TATCAGCTCA | CTCAAAGGCG | GTAATACGGT | TATCCACAGA | ATCAGGGGAT |
| 1660 | 1670 | 1680 | 1690 | 1700 |
| AACGCAGGAA | AGAACATGTG | AGCAAAAGGC | CAGCAAAAGG | CCAGGAACCG |
| 1710 | 1720 | 1730 | 1740 | 1750 |
| TAAAAAGGCC | GCGTTGCTGG | CGTTTTTCCA | TAGGCTCCGC | CCCCCTGACG |
| 1760 | 1770 | 1780 | 1790 | 1800 |
| AGCATCACAA | AAATCGACGC | TCAAGTCAGA | GGTGGCGAAA | CCCGACAGGA |
| 1810 | 1820 | 1830 | 1840 | 1850 |
| CTATAAAGAT | ACCAGGCGTT | TCCCCCTGGA | AGCTCCCTCG | TGCGCTCTCC |

FIGURE 7 (continued)

| | | | | |
|------------|------------|-------------|-------------|-------------|
| 1860 | 1870 | 1880 | 1890 | 1900 |
| TGTTCCGACC | CTGCCGCTTA | CCGGATACCT | GTCCGCCTTT | CTCCCTTCGG |
| 1910 | 1920 | 1930 | 1940 | 1950 |
| GAAGCGTGGC | GCTTTCTCAA | TGCTCACGCT | GTAGGTATCT | CAGTTCGGTG |
| 1960 | 1970 | 1980 | 1990 | 2000 |
| TAGGTCGTTT | GCTCCAAGCT | GGGCTGTGTG | CACGAACCCC | CCGTTTCAGCC |
| 2010 | 2020 | 2030 | 2040 | 2050 |
| CGACCGCTGC | GCCTTATCCG | GTAACATCG | TCTTGAGTCC | AACCCGTA |
| 2060 | 2070 | 2080 | 2090 | 2100 |
| GACACGACTT | ATCGCCACTG | GCAGCAGCCA | CTGGTAACAG | GATTAGCAGA |
| 2110 | 2120 | 2130 | 2140 | 2150 |
| GCGAGGTATG | TAGGCGGTGC | TACAGAGTTC | TTGAAGTGGT | GGCCTAACTA |
| 2160 | 2170 | 2180 | 2190 | 2200 |
| CGGCTACACT | AGAAGGACAG | TATTTGGTAT | CTGCGCTCTG | CTGAAGCCAG |
| 2210 | 2220 | 2230 | 2240 | 2250 |
| TTACCTTCGG | AAAAAGAGTT | GGTAGCTCTT | GATCCGGCAA | ACAAACCACC |
| 2260 | 2270 | 2280 | 2290 | 2300 |
| GCTGGTAGCG | GTGGTTTTTT | TGTTTGCAAG | CAGCAGATTA | CGCGCAGAAA |
| 2310 | 2320 | 2330 | 2340 | 2350 |
| AAAAGGATCT | CAAGAAGATC | CTTTGATCTT | TTCTACGGGG | TCTGACGCTC |
| 2360 | 2370 | 2380 | 2390 | 2400 |
| AGTGGAACGA | AAACTCACGT | TAAGGGATT | TGGTCATGAG | ATTATCAAAA |
| 2410 | 2420 | 2430 | 2440 | 2450 |
| AGGATCTTCA | CCTAGATCCT | TTTAAATTAA | AAATGAAGTT | TTAAATCAAT |
| 2460 | 2470 | 2480 | 2490 | 2500 |
| CTAAAGTATA | TATGAGTAAA | CTTGGTCTGA | CAGTTACCAA | TGCTTAATCA |
| 2510 | 2520 | 2530 | 2540 | 2550 |
| GTGAGGCACC | TATCTCAGCG | ATCTGTCTAT | TTCGTTTCATC | CATAGTTGCC |
| 2560 | 2570 | 2580 | 2590 | 2600 |
| TGACTCCCCG | TCGTGTAGAT | AACTACGATA | CGGGAGGGCT | TACCATCTGG |
| 2610 | 2620 | 2630 | 2640 | 2650 |
| CCCCAGTGCT | GCAATGATAC | CGCGAGACCC | ACGCTCACCG | GCTCCAGATT |
| 2660 | 2670 | 2680 | 2690 | 2700 |
| TATCAGCAAT | AAACCAGCCA | GCCGGAAGGG | CCGAGCGCAG | AAGTGGTCCT |
| 2710 | 2720 | 2730 | 2740 | 2750 |
| GCAACTTTAT | CCGCCTCCAT | CCAGTCTATT | AATTGTTGCC | GGGAAGCTAG |
| 2760 | 2770 | 2780 | 2790 | 2800 |
| AGTAAGTAGT | TCGCCAGTTA | ATAGTTTGCG | CAACGTTGTT | GCCATTGCTG |
| 2810 | 2820 | 2830 | 2840 | 2850 |
| CAGGCATCGT | GGTGTACGCG | TCGTCGTTTG | GTATGGCTTC | ATTCAGCTCC |
| 2860 | 2870 | 2880 | 2890 | 2900 |
| GGTTCCCAAC | GATCAAGGCG | AGTTACATGA | TCCCCCATGT | TGTGCAAAAA |
| 2910 | 2920 | 2930 | 2940 | 2950 |
| AGCGGTTAGC | TCCTTCGGTC | CTCCGATCGT | TGTCAGAAGT | AAGTTGGCCG |
| 2960 | 2970 | 2980 | 2990 | 3000 |
| CAGTGTATC | ACTCATGGTT | ATGGCAGCAC | TGCATAATTC | TCTTACTGTC |
| 3010 | 3020 | 3030 | 3040 | 3050 |
| ATGCCATCCG | TAAGATGCTT | TTCTGTGACT | GGTGAGTACT | CAACCAAGTC |
| 3060 | 3070 | 3080 | 3090 | 3100 |
| ATTCTGAGAA | TAGTGTATGC | GGCGACCGAG | TTGCTCTTGC | CCGGCGTCAA |
| 3110 | 3120 | 3130 | 3140 | 3150 |
| CACGGGATAA | TACCGCGCCA | CATAGCAGAA | CTTTAAAAGT | GCTCATCATT |
| 3160 | 3170 | 3180 | 3190 | 3200 |
| GGAAAACGTT | CTTCGGGGCG | AAAACCTCTCA | AGGATCTTAC | CGCTGTTGAG |

FIGURE 7 (continued)

| | | | | |
|------------|------------|------------|------------|------------|
| 3210 | 3220 | 3230 | 3240 | 3250 |
| ATCCAGTTCG | ATGTAACCCA | CTCGTGCACC | CAACTGATCT | TCAGCATCTT |
| 3260 | 3270 | 3280 | 3290 | 3300 |
| TTACTTTCAC | CAGCGTTTCT | GGGTGAGCAA | AAACAGGAAG | GCAAAATGCC |
| 3310 | 3320 | 3330 | 3340 | 3350 |
| GCAAAAAAGG | GAATAAGGGC | GACACGGAAA | TGTTGAATAC | TCATACTCTT |
| 3360 | 3370 | 3380 | 3390 | 3400 |
| CCTTTTTCAA | TATTATTGAA | GCATTTATCA | GGGTTATTGT | CTCATGAGCG |
| 3410 | 3420 | 3430 | 3440 | 3450 |
| GATACATATT | TGAATGTATT | TAGAAAAATA | AACAAATAGG | GGTTCCGCGC |
| 3460 | 3470 | 3480 | 3490 | 3500 |
| ACATTTCCCC | GAAAAGTGCC | ACCTGACGTC | TAAGAAACCA | TTATTATCAT |
| 3510 | 3520 | 3530 | 3540 | 3550 |
| GACATTAACC | TATAAAAATA | GGCGTATCAC | GAGGCCCTTT | CGTCTTCAAG |

AA

FIGURE 8

| | | | | |
|------------|------------|------------|------------|------------|
| 10 | 20 | 30 | 40 | 50 |
| GTTGACACAT | ATGAGTCTTG | TGATGTACTG | GCTGATTTCT | ACGACCAGTT |
| 60 | 70 | 80 | 90 | 100 |
| CGCTGACCAG | TTGCACGAGT | CTCAATTGGA | CAAAATGCCA | GCACTTCCGG |
| 110 | 120 | 130 | 140 | 150 |
| CTAAAGGTAA | CTTGAACCTC | CGTGACATCT | TAGAGTCGGA | CTTCGCGTTC |
| 160 | 170 | 180 | 190 | 200 |
| GCGTAACGCC | AAATCAATAC | GACTCACTAT | AGAGGGACAA | ACTCAAGGTC |
| 210 | 220 | 230 | 240 | 250 |
| ATTCGCAAGA | GTGGCCTTTA | TGATTGACCT | TCTTCCGGTT | AATACGACTC |
| 260 | 270 | 280 | 290 | 300 |
| ACTATAGGAG | AACCTTAAGG | TTTAACTTTA | AGACCCTTAA | GTGTTAATTA |
| 310 | 320 | 330 | 340 | 350 |
| GAGATTTAAA | TTAAAGAATT | ACTAAGAGAG | GACTTTAAGT | ATGCGTAACT |
| 360 | 370 | 380 | 390 | 400 |
| TCGAAAAGAT | GACCAAACGT | TCTAACCGTA | ATGCTCGTGA | CTTCGAGGCA |
| 410 | 420 | 430 | 440 | 450 |
| ACCAAAGGTC | GCAAGTTGAA | TAAGACTAAG | CGTGACCGCT | CTCACAAGCG |
| 460 | 470 | 480 | 490 | 500 |
| TAGCTGGGAG | GGTCAGTAAG | ATGGGACGTT | TATATAGTGG | TAATCTGGCA |
| 510 | 520 | 530 | 540 | 550 |
| CCGGATCCGG | TATGAAGAGA | TTGTTAAGTC | ACGATAATCA | ATAGGAGAAA |
| 560 | 570 | 580 | 590 | 600 |
| TCAATATGAT | CGTTTCTGAC | ATCGAAGCTA | ACGCCCTCTT | AGAGAGCGTC |

FIGURE 8(continued)

| | | | | |
|------------|------------|------------|------------|------------|
| 610 | 620 | 630 | 640 | 650 |
| ACTAAGTTCC | ACTGCGGGT | TATCTACGAC | TACTCCACCG | CTGAGTACGT |
| 660 | 670 | 680 | 690 | 700 |
| AAGCTACCGT | CCGAGTGA | TCGGTGCGTA | TCTGGATGCG | CTGGAAGCCG |
| 710 | 720 | 730 | 740 | 750 |
| AGGTTGCACG | AGGCGGTCTT | ATTGTGTTCC | ACAACGGTCA | CAAGTATGAC |
| 760 | 770 | 780 | 790 | 800 |
| GTTCTGTCAT | TGACCAAACT | GGCAAAGTTG | CAATTGAACC | GAGAGTTCCA |
| 810 | 820 | 830 | 840 | 850 |
| CCTTCCTCGT | GAGAACTGTA | TTGACACCCT | TGTGTTGTCA | CGTTTGATT |
| 860 | 870 | 880 | 890 | 900 |
| ATTCCAACCT | CAAGGACACC | GATATGGGTC | TTCTGCGTTC | CGGCAAGTTG |
| 910 | 920 | 930 | 940 | 950 |
| CCCGGAAAAC | GCTTTGGGTC | TCACGCTTTG | GAGGCGTGGG | GTTATCGCTT |
| 960 | 970 | 980 | 990 | 1000 |
| AGGCGAGATG | AAGGGTGAAT | ACAAAGACGA | CTTTAAGCGT | ATGCTTGAAG |
| 1010 | 1020 | 1030 | 1040 | 1050 |
| AGCAGGGTGA | AGAATACGTT | GACGGAATGG | AGTGGTGGAA | CTTCAACGAA |
| 1060 | 1070 | 1080 | 1090 | 1100 |
| GAGATGATGG | ACTATAACGT | TCAGGACGTT | GTGGTAACTA | AAGCTCTCCT |
| 1110 | 1120 | 1130 | 1140 | 1150 |
| TGAGAAGCTA | CTCTCTGACA | AACATTACTT | CCCTCCTGAG | ATTGACTTTA |
| 1160 | 1170 | 1180 | 1190 | 1200 |
| CGGACGTAGG | ATACACTACG | TTCTGGTCAG | AATCCCTTGA | GGCCGTTGAC |
| 1210 | 1220 | 1230 | 1240 | 1250 |
| ATTGAACATC | GTGCTGCATG | GCTGCTCGCT | AAACAAGAGC | GCAACGGGTT |
| 1260 | 1270 | 1280 | 1290 | 1300 |
| CCCGTTTGAC | ACAAAAGCAA | TCGAAGAGTT | GTACGTAGAG | TTAGCTGCTC |
| 1310 | 1320 | 1330 | 1340 | 1350 |
| GCCGCTCTGA | GTTGCTCCGT | AAATTGACCG | AAACGTTCCG | CTCGTGGTAT |
| 1360 | 1370 | 1380 | 1390 | 1400 |
| CAGCCTAAAG | GTGGCACTGA | GATGTTCTGC | CATCCGCGAA | CAGGTAAGCC |
| 1410 | 1420 | 1430 | 1440 | 1450 |
| ACTACCTAAA | TACCTCGCA | TTAAGACACC | TAAAGTTGGT | GGTATCTTTA |
| 1460 | 1470 | 1480 | 1490 | 1500 |
| AGAAGCCTAA | GAACAAGGCA | CAGCGAGAAG | GCCGTGAGCC | TTGCGAAGTT |
| 1510 | 1520 | 1530 | 1540 | 1550 |
| GATACCCGCG | AGTACGTTGC | TGGTGCTCCT | TACACCCGAG | TTGAACATGT |
| 1560 | 1570 | 1580 | 1590 | 1600 |
| TGTGTTTAA | CCTTCGTCTC | GTGACCACAT | TCAGAAGAAA | CTCCAAGAGG |
| 1610 | 1620 | 1630 | 1640 | 1650 |
| CTGGGTGGGT | CCCGACCAAG | TACACCGATA | AGGGTGCTCC | TGTGGTGGAC |
| 1660 | 1670 | 1680 | 1690 | 1700 |
| GATGAGGTAC | TCGAAGGAGT | ACGTGTAGAT | GACCCTGAGA | AGCAAGCCGC |
| 1710 | 1720 | 1730 | 1740 | 1750 |
| TATCGACCTC | ATTAAAGAGT | ACTTGATGAT | TCAGAAGCGA | ATCGGACAGT |
| 1760 | 1770 | 1780 | 1790 | 1800 |
| CTGCTGAGGG | AGACAAAGCA | TGGCTTCGTT | ATGTTGCTGA | GGATGGTAAG |
| 1810 | 1820 | 1830 | 1840 | 1850 |
| ATTCATGGTT | CTGTTAACCC | TAATGGAGCA | GTTACGGGTC | GTGCGACCCA |
| 1860 | 1870 | 1880 | 1890 | 1900 |
| TGCGTTCCCA | AACCTTGCGC | AAATTCCGGG | TGTACGTTCT | CCTTATGGAG |
| 1910 | 1920 | 1930 | 1940 | 1950 |
| AGCAGTGTG | CGCTGCTTTT | GGCGCTGAGC | ACCATTGGA | TGGGATAACT |

FIGURE 8 (continued)

| | | | | |
|------------|-------------|-------------|------------|-------------|
| 1960 | 1970 | 1980 | 1990 | 2000 |
| GGTAAGCCTT | GGGTTTCAGGC | TGGCATCGAC | GCATCCGGTC | TTGAGCTACG |
| 2010 | 2020 | 2030 | 2040 | 2050 |
| CTGCTTGGCT | CACTTCATGG | CTCGCTTTGA | TAACGGCGAG | TACGCTCACG |
| 2060 | 2070 | 2080 | 2090 | 2100 |
| AGATTCTTAA | CGGCGACATC | CACACTAAGA | ACCAGATAGC | TGCTGAACCTA |
| 2110 | 2120 | 2130 | 2140 | 2150 |
| CCTACCCGAG | ATAACGCTAA | GACGTTTCATC | TATGGGTTCC | TCTATGGTGC |
| 2160 | 2170 | 2180 | 2190 | 2200 |
| TGGTGATGAG | AAGATTGGAC | AGATTGTTGG | TGCTGGTAAA | GAGCGCGGTA |
| 2210 | 2220 | 2230 | 2240 | 2250 |
| AGGAACTCAA | GAAGAAATTC | CTTGAGAACA | CCCCCGCGAT | TGCAGCACTC |
| 2260 | 2270 | 2280 | 2290 | 2300 |
| CGCGAGTCTA | TCCAACAGAC | ACTTGTCGAG | TCCTCTCAAT | GGGTAGCTGG |
| 2310 | 2320 | 2330 | 2340 | 2350 |
| TGAGCAACAA | GTCAAGTGGA | AACGCCGCTG | GATTAAAGGT | CTGGATGGTC |
| 2360 | 2370 | 2380 | 2390 | 2400 |
| GTAAGGTACA | CGTTCGTAGT | CCTCACGCTG | CCTTGAATAC | CCTACTGCAA |
| 2410 | 2420 | 2430 | 2440 | 2450 |
| TCTGCTGGTG | CTCTCATCTG | CAAACGTGG | ATTATCAAGA | CCGAAGAGAT |
| 2460 | 2470 | 2480 | 2490 | 2500 |
| GCTCGTAGAG | AAAGGCTTGA | AGCATGGCTG | GGATGGGGAC | TTTGCGTACA |
| 2510 | 2520 | 2530 | 2540 | 2550 |
| TGGCATGGGT | ACATGATGAA | ATCCAAGTAG | GCTGCCGTAC | CGAAGAGATT |
| 2560 | 2570 | 2580 | 2590 | 2600 |
| GCTCAGGTGG | TCATTGAGAC | CGCACAAGAA | GCGATGCGCT | GGGTTGGAGA |
| 2610 | 2620 | 2630 | 2640 | 2650 |
| CCACTGGAAC | TTCCGGTGTC | TTCTGGATAC | CGAAGGTAAG | ATGGGTCCTA |
| 2660 | 2670 | 2680 | 2690 | 2700 |
| ATTGGGCGAT | TTGCCACTGA | TACAGGAGGC | TACTCATGAA | CGAAAGACAC |
| 2710 | 2720 | 2730 | 2740 | 2750 |
| TTAACAGGTG | CTGCTTCTGA | AATGCTAGTA | GCCTACAAAT | TTACCAAAGC |
| 2760 | 2770 | 2780 | 2790 | 2800 |
| TGGGTACACT | GTCTATTACC | CTATGCTGAC | TCAGAGTAAA | GAGGACTTGG |
| 2810 | 2820 | 2830 | 2840 | 2850 |
| TTGTATGTAA | GGATGGTAAA | TTTAGTAAGG | TTCAGGTTAA | AACAGCCACA |
| 2860 | 2870 | 2880 | 2890 | 2900 |
| ACGGTTCAAA | CCAACACAGG | AGATGCCAAG | CAGGTTAGGC | TAGGTGGATG |
| 2910 | 2920 | 2930 | 2940 | 2950 |
| CGGTAGGTCC | GAATATAAGG | ATGGAGACTT | TGACATTCTT | GCGGTTGTGG |
| 2960 | 2970 | 2980 | 2990 | 3000 |
| TTGACGAAGA | TGTGCTTATT | TTACATGGG | ACGAAGTAAA | AGGTAAGACA |
| 3010 | 3020 | 3030 | 3040 | 3050 |
| TCCATGTGTG | TCGGCAAGAG | AAACAAAGGC | ATAAACTAT | AGGAGAAATT |
| 3060 | 3070 | 3080 | | |
| ATTATGGCTA | TGACAAAGAA | ATTTCCGGAT | C | |

FIGURE 9

| | | | | |
|------------|------------|------------|------------|------------|
| 10 | 20 | 30 | 40 | 50 |
| AATGCTACTA | CTATTAGTAG | AATTGATGCC | ACCTTTTCAG | CTCGCGCCCC |
| 60 | 70 | 80 | 90 | 100 |
| AAATGAAAAT | ATAGCTAAAC | AGGTTATTGA | CCATTTGCGA | AATGTATCTA |
| 110 | 120 | 130 | 140 | 150 |
| ATGGTCAAAC | TAAATCTACT | CGTTCGCAGA | ATTGGGAATC | AACTGTTACA |
| 160 | 170 | 180 | 190 | 200 |
| TGGAATGAAA | CTTCCAGACA | CCGTACTTTA | GTTGCATATT | TAAAACATGT |
| 210 | 220 | 230 | 240 | 250 |
| TGAGCTACAG | CACCAGATTC | AGCAATTAAG | CTCTAAGCCA | TCCGCAAAAA |
| 260 | 270 | 280 | 290 | 300 |
| TGACCTCTTA | TCAAAAGGAG | CAATTAAAGG | TACTCTCTAA | TCCTGACCTG |
| 310 | 320 | 330 | 340 | 350 |
| TTGGAGTTTG | CTTCCGGTCT | GGTTCGCTTT | GAAGCTCGAA | TTAAAACGCG |
| 360 | 370 | 380 | 390 | 400 |
| ATATTTGAAG | TCTTTCGGGC | TTCCTCTTAA | TCTTTTGTAT | GCAATCCGCT |
| 410 | 420 | 430 | 440 | 450 |
| TTGCTTCTGA | CTATAATAGT | CAGGGTAAAG | ACCTGATTTT | TGATTTATGG |
| 460 | 470 | 480 | 490 | 500 |
| TCATTCTCGT | TTTCTGAACT | GTTTAAAGCA | TTTGAGGGGG | ATTCAATGAA |
| 510 | 520 | 530 | 540 | 550 |
| TATTTATGAC | GATTCCGCAG | TATTGGACGC | TATCCAGTCT | AAACATTTTA |
| 560 | 570 | 580 | 590 | 600 |
| CTATTACCCC | CTCTGGCAAA | ACTTCTTTTG | CAAAAGCCTC | TCGCTATTTT |
| 610 | 620 | 630 | 640 | 650 |
| GGTTTTTATC | GTCGTCTGGT | AAACGAGGGT | TATGATAGTG | TTGCTCTTAC |
| 660 | 670 | 680 | 690 | 700 |
| TATGCCTCGT | AATTCCTTTT | GGCGTTATGT | ATCTGCATTA | GTTGAATGTG |
| 710 | 720 | 730 | 740 | 750 |
| GTATTCCTAA | ATCTCAACTG | ATGAATCTTT | CTACCTGTAA | TAATGTTGTT |
| 760 | 770 | 780 | 790 | 800 |
| CCGTTAGTTC | GTTTTATTAA | CGTAGATTTT | TCTTCCCAAC | GTCCTGACTG |
| 810 | 820 | 830 | 840 | 850 |
| GTATAATGAG | CCAGTTCCTA | AAATCGCATA | AGGTAATTCA | CAATGATTAA |
| 860 | 870 | 880 | 890 | 900 |

FIGURE 9 (continued)

| | | | | |
|-------------|------------|-------------|------------|-------------|
| AGTTGAAATT | AAACCATCTC | AAGCCCAATT | TACTACTCGT | TCTGGTGGTT |
| 910 | 920 | 930 | 940 | 950 |
| CTCGTCAGGG | CAAGCCTTAT | TCACTGAATG | AGCAGCTTTG | TTACGTTGAT |
| 960 | 970 | 980 | 990 | 1000 |
| TTGGGTAATG | AATATCCGGT | TCTTGTC AAG | ATTACTCTTG | ATGAAGGTCA |
| 1010 | 1020 | 1030 | 1040 | 1050 |
| GCCAGCCTAT | GCGCCTGGTC | TGTACACCGT | TCATCTGTCC | TCTTTC AAG |
| 1060 | 1070 | 1080 | 1090 | 1100 |
| TTGGTCAGTT | CGGTTCCCTT | ATGATTGACC | GTCTGCGCCT | CGTTCCGGCT |
| 1110 | 1120 | 1130 | 1140 | 1150 |
| AAGTAACATG | GAGCAGGTCT | CGGATTTCGA | CACAATTTAT | CAGGCGATGA |
| 1160 | 1170 | 1180 | 1190 | 1200 |
| TACAAATCTC | CGTTGTACTT | TGTTTCGCGC | TTGGTATAAT | CGCTGGGGGT |
| 1210 | 1220 | 1230 | 1240 | 1250 |
| CAAAGATGAG | TGTTTTAGTG | TATTCTTTTCG | CCTCTTTCGT | TTTAGGTTGG |
| 1260 | 1270 | 1280 | 1290 | 1300 |
| TGCCTTCGTA | GTGGCATTAC | GTATTTTACC | CGTTTAATGC | AAACTTCCTC |
| 1310 | 1320 | 1330 | 1340 | 1350 |
| ATGAAAAAGT | CTTTAGTCCT | CAAAGCCTCT | GTAGCCGTTG | CTACCCTCGT |
| 1360 | 1370 | 1380 | 1390 | 1400 |
| TCCGATGCTG | TCTTTCGCTG | CTGAGGGTGA | CGATCCCGCA | AAAGCGGCCT |
| 1410 | 1420 | 1430 | 1440 | 1450 |
| TTAACTCCCT | GCAAGCCTCA | GCGACCGAAT | ATATCGGTTA | TGCGTGGGCG |
| 1460 | 1470 | 1480 | 1490 | 1500 |
| ATGGTTGTTG | TCATTGTCGG | CGCAACTATC | GGTATCAAGC | TGTTTAAGAA |
| 1510 | 1520 | 1530 | 1540 | 1550 |
| ATTCACCTCG | AAAGCAAGCT | GATAAACCGA | TACAATTAAA | GGCTCCTTTT |
| 1560 | 1570 | 1580 | 1590 | 1600 |
| GGAGCCTTTT | TTTTTGAGAA | TTTTCAACGT | GAAAAAATTA | TTATTTCGCAA |
| 1610 | 1620 | 1630 | 1640 | 1650 |
| TTCCTTTAGT | TGTTCCTTTC | TATTCTCACT | CCGCTGAAAC | TGTTGAAAGT |
| 1660 | 1670 | 1680 | 1690 | 1700 |
| TGTTTAGCAA | AACCCCATAC | AGAAAATTCA | TTTACTAACG | TCTGGAAAGA |
| 1710 | 1720 | 1730 | 1740 | 1750 |
| CGACAAAAC | TTAGATCGTT | ACGCTAACTA | TGAGGGTTGT | CTGTGGAATG |
| 1760 | 1770 | 1780 | 1790 | 1800 |
| CTACAGGCGT | TGTAGTTTGT | ACTGGTGACG | AAACTCAGTG | TTACGGTACA |
| 1810 | 1820 | 1830 | 1840 | 1850 |
| TGGGTTCCCTA | TTGGGCTTGC | TATCCCTGAA | AATGAGGGTG | GTGGCTCTGA |
| 1860 | 1870 | 1880 | 1890 | 1900 |
| GGGTGGCGGT | TCTGAGGGTG | GCGGTTCTGA | GGGTGGCGGT | ACTAAACCTC |
| 1910 | 1920 | 1930 | 1940 | 1950 |
| CTGAGTACGG | TGATACACCT | ATTCCGGGCT | ATACTTATAT | CAACCCTCTC |
| 1960 | 1970 | 1980 | 1990 | 2000 |
| GACGGCACTT | ATCCGCCTGG | TACTGAGCAA | AACCCCGCTA | ATCCTAATCC |
| 2010 | 2020 | 2030 | 2040 | 2050 |
| TTCTCTTGAG | GAGTCTCAGC | CTCTTAATAC | TTTCATGTTT | CAGAATAATA |
| 2060 | 2070 | 2080 | 2090 | 2100 |
| GGTTCCGAAA | TAGGCAGGGG | GCATTAACTG | TTTATACGGG | CACTGTTACT |
| 2110 | 2120 | 2130 | 2140 | 2150 |
| CAAGGCACTG | ACCCCGTTAA | AACTTATTAC | CAGTACACTC | CTGTATCATC |
| 2160 | 2170 | 2180 | 2190 | 2200 |
| AAAAGCCATG | TATGACGCTT | ACTGGAACGG | TAAATTCAGA | GACTGCGCTT |
| 2210 | 2220 | 2230 | 2240 | 2250 |

FIGURE 9 (continued)

| | | | | |
|------------|------------|-------------|------------|------------|
| TCCATTCTGG | CTTTAATGAA | GATCCATTCTG | TTTGTGAATA | TCAAGGCCAA |
| 2260 | 2270 | 2280 | 2290 | 2300 |
| TCGTCTGACC | TGCCTCAACC | TCCTGTCAAT | GCTGGCGGCG | GCTCTGGTGG |
| 2310 | 2320 | 2330 | 2340 | 2350 |
| TGGTTCTGGT | GGCGGCTCTG | AGGGTGGTGG | CTCTGAGGGT | GGCGGTTCTG |
| 2360 | 2370 | 2380 | 2390 | 2400 |
| AGGGTGGCGG | CTCTGAGGGA | GGCGGTTCCG | GTGGTGGCTC | TGGTTCCGGT |
| 2410 | 2420 | 2430 | 2440 | 2450 |
| GATTTTGATT | ATGAAAAGAT | GGCAAACGCT | AATAAGGGGG | CTATGACCGA |
| 2460 | 2470 | 2480 | 2490 | 2500 |
| AAATGCCGAT | GAAAACGCGC | TACAGTCTGA | CGCTAAAGGC | AAACTTGATT |
| 2510 | 2520 | 2530 | 2540 | 2550 |
| CTGTCGCTAC | TGATTACGGT | GCTGCTATCG | ATGGTTTCAT | TGGTGACGTT |
| 2560 | 2570 | 2580 | 2590 | 2600 |
| TCCGGCCTTG | CTAATGGTAA | TGGTGCTACT | GGTGATTTTG | CTGGCTCTAA |
| 2610 | 2620 | 2630 | 2640 | 2650 |
| TTCCCAAATG | GCTCAAGTCG | GTGACGGTGA | TAATTCACCT | TTAATGAATA |
| 2660 | 2670 | 2680 | 2690 | 2700 |
| ATTTCCGTCA | ATATTTACCT | TCCCTCCCTC | AATCGGTTGA | ATGTCGCCCT |
| 2710 | 2720 | 2730 | 2740 | 2750 |
| TTTGTCTTTA | GCGCTGGTAA | ACCATATGAA | TTTTCTATTG | ATTGTGACAA |
| 2760 | 2770 | 2780 | 2790 | 2800 |
| AATAAACTTA | TTCCGTGGTG | TCTTTGCGTT | TCTTTTATAT | GTTGCCACCT |
| 2810 | 2820 | 2830 | 2840 | 2850 |
| TTATGTATGT | ATTTTCTACG | TTTGCTAACA | TACTGCGTAA | TAAGGAGTCT |
| 2860 | 2870 | 2880 | 2890 | 2900 |
| TAATCATGCC | AGTTCTTTTG | GGTATTCGGT | TATTATTGCG | TTTCCTCGGT |
| 2910 | 2920 | 2930 | 2940 | 2950 |
| TTCTTCTGG | TAACCTTTGT | CGGCTATCTG | CTTACTTTTC | TTAAAAAGGG |
| 2960 | 2970 | 2980 | 2990 | 3000 |
| CTTCGGTAAG | ATAGCTATTG | CTATTTTCATT | GTTTCTTGCT | CTTATTATTG |
| 3010 | 3020 | 3030 | 3040 | 3050 |
| GGCTTAACTC | AATTCTTGTC | GGTTATCTCT | CTGATATTAG | CGCTCAATTA |
| 3060 | 3070 | 3080 | 3090 | 3100 |
| CCCTCTGACT | TTGTTCAAGG | TGTTCAAGTA | ATTCTCCCGT | CTAATGCGCT |
| 3110 | 3120 | 3130 | 3140 | 3150 |
| TCCCTGTTTT | TATGTTATTC | TCTCTGTAAA | GGCTGCTATT | TTCATTTTTG |
| 3160 | 3170 | 3180 | 3190 | 3200 |
| ACGTAAACA | AAAAATCGTT | TCTTATTTGG | ATTGGGATAA | ATAATATGGC |
| 3210 | 3220 | 3230 | 3240 | 3250 |
| TGTTTATTTT | GTAAGTGGCA | AATTAGGCTC | TGGAAAGACG | CTCGTTAGCG |
| 3260 | 3270 | 3280 | 3290 | 3300 |
| TTGGTAAGAT | TCAGGATAAA | ATTGTAGCTG | GGTGCAAAAT | AGCAACTAAT |
| 3310 | 3320 | 3330 | 3340 | 3350 |
| CTTGATTTAA | GGCTTCAAAA | CCTCCCGCAA | GTCGGGAGGT | TCGCTAAAAC |
| 3360 | 3370 | 3380 | 3390 | 3400 |
| GCCTCGCGTT | CTTAGAATAC | CGGATAAGCC | TTCTATATCT | GATTTGCTTG |
| 3410 | 3420 | 3430 | 3440 | 3450 |
| CTATTGGGCG | CGGTAATGAT | TCCTACGATG | AAAATAAAAA | CGGCTTGCTT |
| 3460 | 3470 | 3480 | 3490 | 3500 |
| GTTCTCGATG | AGTGCGGTAC | TTGGTTTAAT | ACCCGTTCTT | GGAATGATAA |
| 3510 | 3520 | 3530 | 3540 | 3550 |
| GGAAAGACAG | CCGATTATTG | ATTGGTTTCT | ACATGCTCGT | AAATTAGTAT |
| 3560 | 3570 | 3580 | 3590 | 3600 |

FIGURE 9 (continued)

| | | | | |
|-------------|-------------|------------|------------|------------|
| GGGATATTAT | TTTTCTTGTT | CAGGACTTAT | CTATTGTTGA | TAAACAGGCG |
| 3610 | 3620 | 3630 | 3640 | 3650 |
| CGTTCTGTCAT | TAGCTGAACA | TGTTGTTTAT | TGTCGTCGTC | TGGACAGAAT |
| 3660 | 3670 | 3680 | 3690 | 3700 |
| TACTTTACCT | TTTGTCGGTA | CTTTATATTC | TCTTATTACT | GGCTCGAAAA |
| 3710 | 3720 | 3730 | 3740 | 3750 |
| TGCCTCTGCC | TAAATTACAT | GTTGGCGTTG | TTAAATATGG | CGATTCTCAA |
| 3760 | 3770 | 3780 | 3790 | 3800 |
| TTAAGCCCTA | CTGTTGAGCG | TTGGCTTTAT | ACTGGTAAGA | ATTTGTATAA |
| 3810 | 3820 | 3830 | 3840 | 3850 |
| CGCATATGAT | ACTAAACAGG | CTTTTTCTAG | TAATTATGAT | TCCGGTGTTT |
| 3860 | 3870 | 3880 | 3890 | 3900 |
| ATTCTTATTT | AACGCCCTTAT | TTATCACACG | GTCGGTATTT | CAAACCATTA |
| 3910 | 3920 | 3930 | 3940 | 3950 |
| AATTTAGGTC | AGAAGATGAA | ATTAATAAAA | ATATATTTGA | AAAAGTTTTT |
| 3960 | 3970 | 3980 | 3990 | 4000 |
| TCGCGTTCTT | TGTCTTGCGA | TTGGATTTCG | ATCAGCATTT | ACATATAGTT |
| 4010 | 4020 | 4030 | 4040 | 4050 |
| ATATAACCCA | ACCTAAGCCG | GAGGTTAAAA | AGGTAGTCTC | TCAGACCTAT |
| 4060 | 4070 | 4080 | 4090 | 4100 |
| GATTTTGATA | AATTCACAT | TGACTCTTCT | CAGCGTCTTA | ATCTAAGCTA |
| 4110 | 4120 | 4130 | 4140 | 4150 |
| TCGCTATGTT | TTCAAGGATT | CTAAGGGAAA | ATTAATTAAT | AGCGACGATT |
| 4160 | 4170 | 4180 | 4190 | 4200 |
| TACAGAAGCA | AGGTTATTCA | CTCACATATA | TTGATTTATG | TACTGTTTCC |
| 4210 | 4220 | 4230 | 4240 | 4250 |
| ATTAAAAAAG | GTAATTCAAA | TGAAATTGTT | AAATGTAATT | AATTTTGTTT |
| 4260 | 4270 | 4280 | 4290 | 4300 |
| TCTTGATGTT | TGTTTCATCA | TCTTCTTTTG | CTCAGGTAAT | TGAAATGAAT |
| 4310 | 4320 | 4330 | 4340 | 4350 |
| AATTCGCCTC | TGCGCGATTT | TGTAACCTGG | TATTCAAAGC | AATCAGGCGA |
| 4360 | 4370 | 4380 | 4390 | 4400 |
| ATCCGTTTAT | GTTTCTCCCG | ATGTAAAAGG | TACTGTACT | GTATATTCAT |
| 4410 | 4420 | 4430 | 4440 | 4450 |
| CTGACGTTAA | ACTTGAAAAT | CTACGCAATT | TCTTTATTTT | TGTTTTACGT |
| 4460 | 4470 | 4480 | 4490 | 4500 |
| GCTAATAATT | TTGATATGGT | TGGTTCAATT | CCTTCCATAA | TTCAGAAGTA |
| 4510 | 4520 | 4530 | 4540 | 4550 |
| TAATCCAAAC | AATCAGGTAT | ATATTGATGA | ATTGCCATCA | TCTGATAATC |
| 4560 | 4570 | 4580 | 4590 | 4600 |
| AGGAATATGA | TGATAATTCC | GCTCCTTCTG | GTGGTTTCTT | TGTTCCGCAA |
| 4610 | 4620 | 4630 | 4640 | 4650 |
| AATGATAATG | TTACTCAAAC | TTTTAAAATT | AATAACGTTT | GGGCAAAGGA |
| 4660 | 4670 | 4680 | 4690 | 4700 |
| TTTAATACGA | GTTGTCGAAT | TGTTTGTAAT | GTCTAATACT | TCTAAATCCT |
| 4710 | 4720 | 4730 | 4740 | 4750 |
| CAAATGTATT | ATCTATTGAC | GGCTCTAATC | TATTAGTTGT | TAGTGCACCT |
| 4760 | 4770 | 4780 | 4790 | 4800 |
| AAAGATATTT | TAGATAACCT | TCCTCAATTC | CTTTCTACTG | TTGATTTGCC |
| 4810 | 4820 | 4830 | 4840 | 4850 |
| AAC TGACCAG | ATATTGATTG | AGGGTTTGAT | ATTTGAGGTT | CAGCAAGGTG |
| 4860 | 4870 | 4880 | 4890 | 4900 |
| ATGCTTTAGA | TTTTTCATTT | GCTGCTGGCT | CTCAGCGTGG | CACTGTTGCA |
| 4910 | 4920 | 4930 | 4940 | 4950 |

FIGURE 9 (continued)

| | | | | |
|-------------|-------------|------------|------------|-------------|
| GGCGGTGTTA | ATACTGACCG | CCTCACCTCT | GTTTTATCTT | CTGCTGGTGG |
| 4960 | 4970 | 4980 | 4990 | 5000 |
| TTCGTTCCGGT | ATTTTAAATG | GCGATGTTTT | AGGGCTATCA | GTTCCGCGCAT |
| 5010 | 5020 | 5030 | 5040 | 5050 |
| TAAAGACTAA | TAGCCATTCA | AAAATATTGT | CTGTGCCACG | TATTCTTACG |
| 5060 | 5070 | 5080 | 5090 | 5100 |
| CTTTCAGGTC | AGAAGGGTTC | TATCTCTGTT | GGCCAGAATG | TCCCTTTTAT |
| 5110 | 5120 | 5130 | 5140 | 5150 |
| TACTGGTCGT | GTGACTGGTG | AATCTGCCAA | TGTAAATAAT | CCATTTCAGA |
| 5160 | 5170 | 5180 | 5190 | 5200 |
| CGATTGAGCG | TCAAAATGTA | GGTATTTCCA | TGAGCGTTTT | TCCTGTTGCA |
| 5210 | 5220 | 5230 | 5240 | 5250 |
| ATGGCTGGCG | GTAATATTGT | TCTGGATATT | ACCAGCAAGG | CCGATAGTTT |
| 5260 | 5270 | 5280 | 5290 | 5300 |
| GAGTCTTCT | ACTCAGGCAA | GTGATGTTAT | TACTAATCAA | AGAAGTATTG |
| 5310 | 5320 | 5330 | 5340 | 5350 |
| CTACAACGGT | TAATTTGCGT | GATGGACAGA | CTCTTTTACT | CGGTGGCCTC |
| 5360 | 5370 | 5380 | 5390 | 5400 |
| ACTGATTATA | AAAACACTTC | TCAAGATTCT | GGCGTACCGT | TCCTGTCTAA |
| 5410 | 5420 | 5430 | 5440 | 5450 |
| AATCCCTTTA | ATCGGCCTCC | TGTTTAGCTC | CCGCTCTGAT | TCCAACGAGG |
| 5460 | 5470 | 5480 | 5490 | 5500 |
| AAAGCACGTT | ATACGTGCTC | GTCAAAGCAA | CCATAGTACG | CGCCCTGTAG |
| 5510 | 5520 | 5530 | 5540 | 5550 |
| CGGCGCATT | AGCGCGGCGG | GTGTGGTGGT | TACGCGCAGC | GTGACCGCTA |
| 5560 | 5570 | 5580 | 5590 | 5600 |
| CACTTGCCAG | CGCCCTAGCG | CCCGCTCCTT | TCGCTTTCTT | CCCTTCCTTT |
| 5610 | 5620 | 5630 | 5640 | 5650 |
| CTCGCCACGT | TCGCCGGCTT | TCCCGTCAA | GCTCTAAATC | GGGGGCTCCC |
| 5660 | 5670 | 5680 | 5690 | 5700 |
| TTTAGGGTTC | CGATTTAGTG | CTTTACGGCA | CCTCGACCCC | AAAAAATTG |
| 5710 | 5720 | 5730 | 5740 | 5750 |
| ATTTGGGTGA | TGGTTCACGT | AGTGGGCCAT | CGCCCTGATA | GACGGTTTTT |
| 5760 | 5770 | 5780 | 5790 | 5800 |
| CGCCCTTTGA | CGTTGGAGTC | CACGTTCTTT | AATAGTGGAC | TCTTGTTCCA |
| 5810 | 5820 | 5830 | 5840 | 5850 |
| AACTGGAACA | ACACTCAACC | CTATCTCGGG | CTATTCCTTT | GATTTATAAG |
| 5860 | 5870 | 5880 | 5890 | 5900 |
| GGATTTTGCC | GATTTTCGGAA | CCACCATCAA | ACAGGATTTT | CGCCTGCTGG |
| 5910 | 5920 | 5930 | 5940 | 5950 |
| GGCAAACCAG | CGTGGACCGC | TTGCTGCAAC | TCTCTCAGGG | CCAGGCGGTG |
| 5960 | 5970 | 5980 | 5990 | 6000 |
| AAGGGCAATC | AGCTGTTGCC | CGTCTCGCTG | GTGAAAAGAA | AAACCACCCT |
| 6010 | 6020 | 6030 | 6040 | 6050 |
| GGCGCCCAAT | ACGCAAACCG | CCTCTCCCCG | CGCGTTGGCC | GATTCATTAA |
| 6060 | 6070 | 6080 | 6090 | 6100 |
| TCCAGCTGGC | ACGACAGGTT | TCCCGACTGG | AAAGCGGGCA | GTGAGCGCAA |
| 6110 | 6120 | 6130 | 6140 | 6150 |
| CGCAATTAAT | GTGAGTIACC | TCACTCATT | GGCACCACAG | GCTTTTACACT |
| 6160 | 6170 | 6180 | 6190 | 6200 |
| TTATGCTTCC | GGCTCGTATG | TTGTGTGGAA | TTGTGAGCGG | ATAACAATTT |
| 6210 | 6220 | 6230 | 6240 | 6250 |
| CACACAGGAA | ACAGCTATGA | CCATGATTAC | GAATTCGAGC | TCGCCCCGGG |
| 6260 | 6270 | 6280 | 6290 | 6300 |

FIGURE 9 (continued)

| | | | | |
|------------|------------|------------|------------|-------------|
| ATCTGCCTGA | ATAGGTACGA | TTTACTAACT | GGAAGAGGCA | CTAAATGAAC |
| 6310 | 6320 | 6330 | 6340 | 6350 |
| ACGATTAACA | TCGCTAAGAA | CGACTTCTCT | GACATCGAAC | TGGCTGCTAT |
| 6360 | 6370 | 6380 | 6390 | 6400 |
| CCCCTTCAAC | ACTCTGGCTG | ACCATTACGG | TGAGCGTTTA | GCTCGCGAAC |
| 6410 | 6420 | 6430 | 6440 | 6450 |
| AGTTGGCCCT | TGAGCATGAG | TCTTACGAGA | TGGGTGAAGC | ACGCTTCCGC |
| 6460 | 6470 | 6480 | 6490 | 6500 |
| AAGATGTTTG | AGCGTCAACT | TAAAGCTGGT | GAGGTTGCGG | ATAACGCTGC |
| 6510 | 6520 | 6530 | 6540 | 6550 |
| CGCCAAGCCT | CTCATCACTA | CCCTACTCCC | TAAGATGATT | GCACGCATCA |
| 6560 | 6570 | 6580 | 6590 | 6600 |
| ACGACTGGTT | TGAGGAAGTG | AAAGCTAAGC | GCGGCAAGCG | CCCGACAGCC |
| 6610 | 6620 | 6630 | 6640 | 6650 |
| TTCCAGTTCC | TGCAAGAAAT | CAAGCCGGAA | GCCGTAGCGT | ACATCACCAT |
| 6660 | 6670 | 6680 | 6690 | 6700 |
| TAAGACCACT | CTGGCTTGCC | TAACCAGTGC | TGACAATACA | ACCGTTCAGG |
| 6710 | 6720 | 6730 | 6740 | 6750 |
| CTGTAGCAAG | CGCAATCGGT | CGGGCCATTG | AGGACGAGGC | TCGCTTCGGT |
| 6760 | 6770 | 6780 | 6790 | 6800 |
| CGTATCCGTG | ACCTTGAAGC | TAAGCACTTC | AAGAAAAACG | TTGAGGAACA |
| 6810 | 6820 | 6830 | 6840 | 6850 |
| ACTCAACAAG | CGCGTAGGGC | ACGTCTACAA | GAAAGCATTT | ATGCAAGTTG |
| 6860 | 6870 | 6880 | 6890 | 6900 |
| TCGAGGCTGA | CATGCTCTCT | AAGGGTCTAC | TCGGTGGCGA | GGCGTGGTCT |
| 6910 | 6920 | 6930 | 6940 | 6950 |
| TCGTGGCATA | AGGAAGACTC | TATTCATGTA | GGAGTACGCT | GCATCGAGAT |
| 6960 | 6970 | 6980 | 6990 | 7000 |
| GCTCATTGAG | TCAACCGGAA | TGGTTAGCTT | ACACCGCCAA | AATGCTGGCG |
| 7010 | 7020 | 7030 | 7040 | 7050 |
| TAGTAGGTCA | AGACTCTGAG | ACTATCGAAC | TCGCACCTGA | ATACGCTGAG |
| 7060 | 7070 | 7080 | 7090 | 7100 |
| GCTATCGCAA | CCCGTGCAGG | TGCGCTGGCT | GGCATCTCTC | CGATGTTCCA |
| 7110 | 7120 | 7130 | 7140 | 7150 |
| ACCTTGCCTA | GTTCTCCTTA | AGCCGTGGAC | TGGCATTACT | GGTGGTGGCT |
| 7160 | 7170 | 7180 | 7190 | 7200 |
| ATTGGGCTAA | CGGTCGTCGT | CCTCTGGCGC | TGGTGCGTAC | TCACAGTAAG |
| 7210 | 7220 | 7230 | 7240 | 7250 |
| AAAGCACTGA | TGCGCTACGA | AGACGTTTAC | ATGCCTGAGG | TGTACAAAGC |
| 7260 | 7270 | 7280 | 7290 | 7300 |
| GATTAACATT | GCGCAAAACA | CCGCATGGAA | AATCAACAAG | AAAGTCCTAG |
| 7310 | 7320 | 7330 | 7340 | 7350 |
| CGGTCGCCAA | CGTAATCACC | AAGTGGAAGC | ATTGTCCGGT | CGAGGACATC |
| 7360 | 7370 | 7380 | 7390 | 7400 |
| CCTGCGATTG | AGCGTGAAGA | ACTCCCGATG | AAACCGGAAG | ACATCGACAT |
| 7410 | 7420 | 7430 | 7440 | 7450 |
| GAATCCTGAG | GCTCTCACCG | CGTGGAACG | TGCTGCCGCT | GCTGTGTACC |
| 7460 | 7470 | 7480 | 7490 | 7500 |
| GCAAGGACAA | GGCTCGCAAG | TCTCGCCGTA | TCAGCCTTGA | GTTTCATGCTT |
| 7510 | 7520 | 7530 | 7540 | 7550 |
| GAGCAAGCCA | ATAAGTTTGC | TAACCATAAG | GCCATCTGGT | TCCCTTACAA |
| 7560 | 7570 | 7580 | 7590 | 7600 |
| CATGGACTGG | CGCGGTCGTG | TTTACGCTGT | GTCAATGTTC | AACCCGCAAG |
| 7610 | 7620 | 7630 | 7640 | 7650 |

FIGURE 9 (continued)

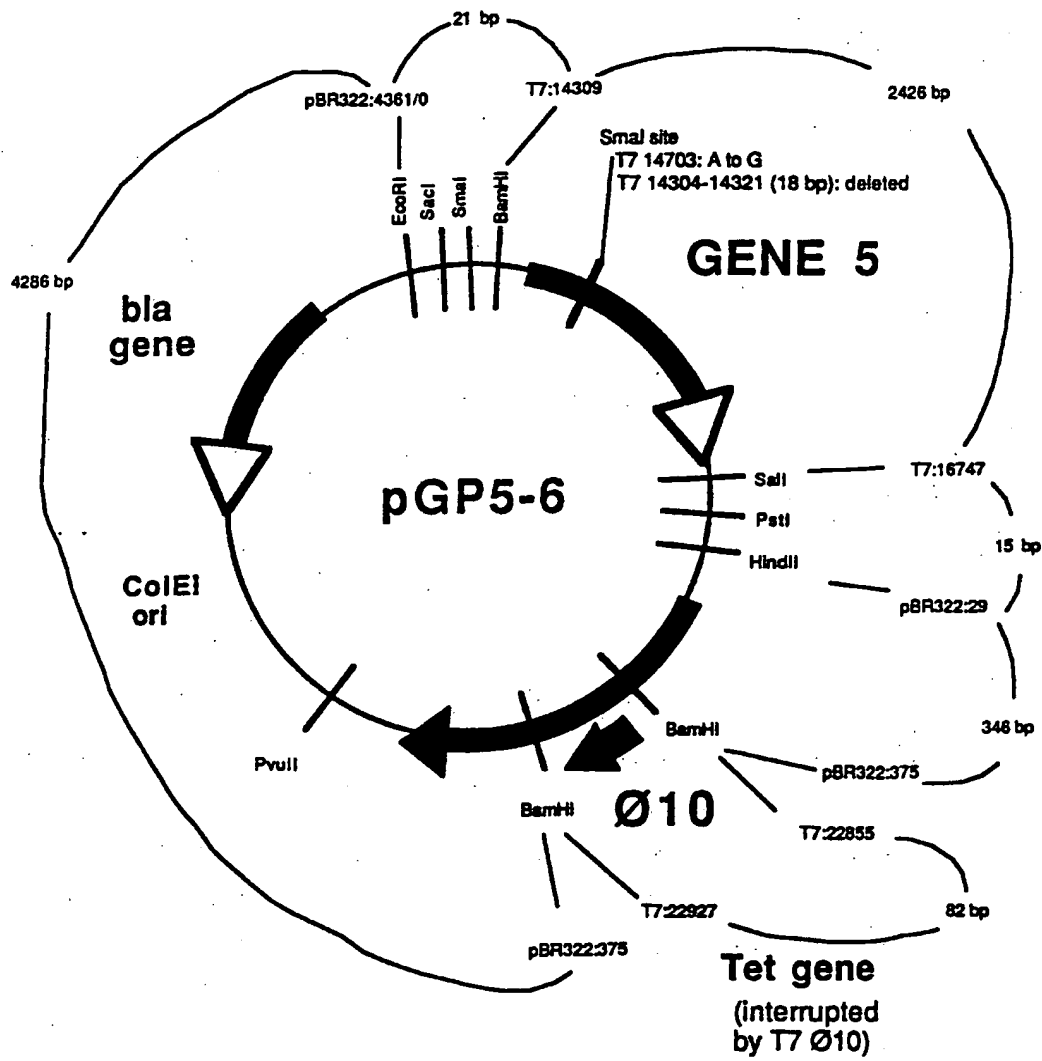
| | | | | |
|------------|------------|-------------|-------------|-------------|
| GTAACGATAT | GACCAAAGGA | CTGCTTACGC | TGGCGAAAGG | TAAACCAATC |
| 7660 | 7670 | 7680 | 7690 | 7700 |
| GGTAAGGAAG | GTTACTACTG | GCTGAAAATC | CACGGTGCAA | ACTGTGCGGG |
| 7710 | 7720 | 7730 | 7740 | 7750 |
| TGTCGATAAG | GTTCCGTTCC | CTGAGCGCAT | CAAGTTCATT | GAGGAAAACC |
| 7760 | 7770 | 7780 | 7790 | 7800 |
| ACGAGAACAT | CATGGCTTGC | GCTAAGTCTC | CACTGGAGAA | CACTTGGTGG |
| 7810 | 7820 | 7830 | 7840 | 7850 |
| GCTGAGCAAG | ATTCTCCGTT | CTGCTTCCTT | GCGTTCCTGCT | TTGAGTACGC |
| 7860 | 7870 | 7880 | 7890 | 7900 |
| TGGGGTACAG | CACCACGGCC | TGAGCTATAA | CTGCTCCCTT | CCGCTGGCGT |
| 7910 | 7920 | 7930 | 7940 | 7950 |
| TTGACGGGTC | TTGCTCTGGC | ATCCAGCACT | TCTCCGCGAT | GCTCCGAGAT |
| 7960 | 7970 | 7980 | 7990 | 8000 |
| GAGGTAGGTG | GTCGCGCGGT | TAACCTTGCTT | CCTAGTGAAA | CCGTTCAGGA |
| 8010 | 8020 | 8030 | 8040 | 8050 |
| CATCTACGGG | ATTGTTGCTA | AGAAAGTCAA | CGAGATTCTA | CAAGCAGACG |
| 8060 | 8070 | 8080 | 8090 | 8100 |
| CAATCAATGG | GACCGATAAC | GAAGTAGTTA | CCGTGACCGA | TGAGAACTACT |
| 8110 | 8120 | 8130 | 8140 | 8150 |
| GGTGAAATCT | CTGAGAAAGT | CAAGCTGGGC | ACTAAGGCAC | TGGCTGGTCA |
| 8160 | 8170 | 8180 | 8190 | 8200 |
| ATGGCTGGCT | TACGGTGTTA | CTCGCAGTGT | GACTAAGCGT | TCAGTCATGA |
| 8210 | 8220 | 8230 | 8240 | 8250 |
| CGCTGGCTTA | CGGGTCCAAA | GAGTTCGGCT | TCCGTCAACA | AGTGCTGGAA |
| 8260 | 8270 | 8280 | 8290 | 8300 |
| GATACCATTC | AGCCAGCTAT | TGATTCCGGC | AAGGGTCTGA | TGTTCACTCA |
| 8310 | 8320 | 8330 | 8340 | 8350 |
| GCCGAATCAG | GCTGCTGGAT | ACATGGCTAA | GCTGATTTGG | GAATCTGTGA |
| 8360 | 8370 | 8380 | 8390 | 8400 |
| GCGTGACGGT | GGTAGCTGCG | GTTGAAGCAA | TGAACTGGCT | TAAGTCTGCT |
| 8410 | 8420 | 8430 | 8440 | 8450 |
| GCTAAGCTGC | TGGCTGCTGA | GGTCAAAGAT | AAGAAGACTG | GAGAGATTCT |
| 8460 | 8470 | 8480 | 8490 | 8500 |
| TCGCAAGCGT | TGCGCTGTGC | ATTGGGTAAC | TCCTGATGGT | TTCCCTGTGT |
| 8510 | 8520 | 8530 | 8540 | 8550 |
| GGCAGGAATA | CAAGAAGCCT | ATTCAGACGC | GCTTGAACCT | GATGTTCCCTC |
| 8560 | 8570 | 8580 | 8590 | 8600 |
| GGTCAGTTCC | GCTTACAGCC | TACCATTAAAC | ACCAACAAAG | ATAGCGAGAT |
| 8610 | 8620 | 8630 | 8640 | 8650 |
| TGATGCACAC | AAACAGGAGT | CTGGTATCGC | TCCTAACTTT | GTACACAGCC |
| 8660 | 8670 | 8680 | 8690 | 8700 |
| AAGACGGTAG | CCACCTTCGT | AAGACTGTAG | TGTGGGCACA | CGAGAAGTAC |
| 8710 | 8720 | 8730 | 8740 | 8750 |
| GGAATCGAAT | CTTTTGCACT | GATTACAGAC | TCCTTCGGTA | CCATTCCGGC |
| 8760 | 8770 | 8780 | 8790 | 8800 |
| TGACGCTGCG | AACCTGTTCA | AAGCAGTGCG | CGAAACTATG | GTTGACACAT |
| 8810 | 8820 | 8830 | 8840 | 8850 |
| ATGAGTCTTG | TGATGTACTG | GCTGATTTCT | ACGACCAGTT | CGCTGACCAG |
| 8860 | 8870 | 8880 | 8890 | 8900 |
| TTGCACGAGT | CTCAATTGGA | CAAAATGCCA | GCACTTCCGG | CTAAAGGTAA |
| 8910 | 8920 | 8930 | 8940 | 8950 |
| CTTGAACCTC | CGTGACATCT | TAGAGTCGGA | CTTCGCGTTC | GCGTAACGCC |
| 8960 | 8970 | 8980 | 8990 | 9000 |

FIGURE 9 (continued)

| | | | | |
|------------|------------|------------|-------------|------------|
| AAATCAATAC | GACCCGGATC | GGTCGACCTG | CAGCCCCAAGC | TTGGCACTGG |
| 9010 | 9020 | 9030 | 9040 | 9050 |
| CCGTCGTTTT | ACAACGTCGT | GACTGGGAAA | ACCCTGGCGT | TACCCAACTT |
| 9060 | 9070 | 9080 | 9090 | 9100 |
| AATCGCCTTG | CAGCACATCC | CCCCTTCGCC | AGCTGGCGTA | ATAGCGAAGA |
| 9110 | 9120 | 9130 | 9140 | 9150 |
| GGCCCGCACC | GATCGCCCTT | CCCAACAGTT | GCGTAGCCTG | AATGGCGAAT |
| 9160 | 9170 | 9180 | 9190 | 9200 |
| GGCGCTTTGC | CTGGTTTCCG | GCACCAGAAG | CGGTGCCGGA | AAGCTGGCTG |
| 9210 | 9220 | 9230 | 9240 | 9250 |
| GAGTGCGATC | TTCCTGAGGC | CGAQACNGTC | GTCGTCCCCT | CAAAGTGGCA |
| 9260 | 9270 | 9280 | 9290 | 9300 |
| GATGCACGGT | TACGATGCGC | CCATCTACAC | CAACGTAACC | TATCCCATTA |
| 9310 | 9320 | 9330 | 9340 | 9350 |
| CGGTCAATCC | GCCGTTTGTT | CCCACGGAGA | ATCCGACGGG | TTGTTACTCG |
| 9360 | 9370 | 9380 | 9390 | 9400 |
| CTCACATTTA | ATGTTGATGA | AAGCTGGCTA | CAGGAAGGCC | AGACGCGAAT |
| 9410 | 9420 | 9430 | 9440 | 9450 |
| TATTTTTGAT | GGCGTTCCTA | TTGGTTAAAA | AATGAGCTGA | TTTAACAAAA |
| 9460 | 9470 | 9480 | 9490 | 9500 |
| ATTTAACGCG | AATTTTAACA | AAATATTAAC | GTTTACAATT | TAAATATTTG |
| 9510 | 9520 | 9530 | 9540 | 9550 |
| CTTATACAAT | CTTCCTGTTT | TTGGGGCTTT | TCTGATTATC | AACCGGGGTA |
| 9560 | 9570 | 9580 | 9590 | 9600 |
| CATATGATTG | ACATGCTAGT | TTTACGATTA | CCGTTTCATCG | ATTCTCTTGT |
| 9610 | 9620 | 9630 | 9640 | 9650 |
| TTGCTCCAGA | CTCTCAGGCA | ATGACCTGAT | AGCCTTTGTA | GATCTCTCAA |
| 9660 | 9670 | 9680 | 9690 | 9700 |
| AAATAGCTAC | CCTCTCCGGC | ATGAATTTAT | CAGCTAGAAC | GGTTGAATAT |
| 9710 | 9720 | 9730 | 9740 | 9750 |
| CATATTGATG | GTGATTGAC | TGTCTCCGGC | CTTTCTCACC | CTTTTGAATC |
| 9760 | 9770 | 9780 | 9790 | 9800 |
| TTTACCTACA | CATTACTCAG | GCATTGCATT | TAAAATATAT | GAGGGTTCTA |
| 9810 | 9820 | 9830 | 9840 | 9850 |
| AAAATTTTAA | TCCTTGCGTT | GAAATAAAGG | CTTCTCCCGC | AAAAGTATTA |
| 9860 | 9870 | 9880 | 9890 | 9900 |
| CAGGGTCATA | ATGTTTTTGG | TACAACCGAT | TTAGCTTTAT | GCTCTGAGGC |
| 9910 | 9920 | 9930 | 9940 | 9950 |
| TTTATTGCTT | AATTTTGCTA | ATTCTTTGCC | TTGCCTGTAT | GATTTATTGG |

ATGTT

FIGURE 10





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 20 2037

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| | | | C12Q |
| The present search report has been drawn up for all claims | | | |
| Place of search THE HAGUE | | Date of completion of the search 14 AUGUST 1992 | Examiner HITCHEN C. E. |
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| X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document | | T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document | |